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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

IMPERIAL COLLEGE INNOVATIONS LIMITED
SHERFIELD BUILDING
IMPERIAL COLLEGE
LONDON
SW7 2AZ

Patents ADP number *(if you know it)*

7409436004

If the applicant is a corporate body, give the country/state of its incorporation

UNITED KINGDOM

4. Title of the invention

SCREENING FOR MODULATORS OF FAT STORAGE

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Carpmaels & Ransford

43 Bloomsbury Square
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WC1A 2RA

Patents ADP number *(if you know it)*

83001

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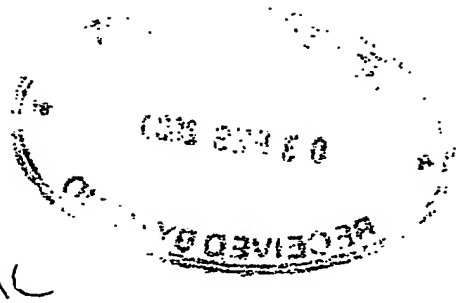

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Cameron Marshall **020-7242 8692**

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SCREENING FOR MODULATORS OF FAT STORAGE

All documents cited herein are incorporated by reference in their entirety

TECHNICAL FIELD

This invention relates to screening methods for identifying compounds that are useful as modulators of fat storage. In particular, the invention provides methods of screening for compounds that modulate the function of receptor interacting protein 140 (RIP140).

BACKGROUND ART

Conditions which involve a significant increase in fat storage, such as obesity, and conditions which involve a significant decrease in fat storage, such as anorexia, are associated with severe health problems. Obesity is associated with diseases such as diabetes, heart disease and hypertension while anorexia can cause irreversible bone damage and ultimately death.

In the past, such conditions have been primarily identified as having psychological causes. However, there is increasing recognition that genetic mechanisms are also involved in conditions where there is a significant deviation in fat storage (Schalling *et al,* J Intern Med 1999 Jun; 245(6): 613-9; Spiegelman & Flier, Cell 2001 Feb; 104: 531-543). For example, the *ob* gene has been suggested to be essential to the control of fat storage and it has been suggested that the leptin polypeptide encoded by the *ob* gene could be used to treat obesity. However, transgenic mouse models used to test the effect of different genes associated with the modulation of fat storage have not always had the expected phenotype (Arch, J Endocrinol Invest 2002 Nov; 25(10):867-75). There is therefore a need to identify further compounds that modulate fat storage. Thus, an object of the invention is the provision of a method for identifying compounds that modulate fat storage.

DISCLOSURE OF THE INVENTION

The nuclear receptor interacting protein 140 (RIP140), also known as Nrip1 (nuclear receptor interacting protein 1), is a co-activator or co-repressor of transcription by a variety of nuclear receptor family members including the estrogen receptors (ER), retinoic acid receptors (RAR), thyroid hormone receptors (TR), retinoid X receptors (RXR), vitamin D receptor (VDR) and peroxisome proliferator activated receptors (PPARá, PPARâ and PPARä), as well as by the aryl hydrocarbon receptor (AhR). It has been also been found to modulate transcription of the steroidogenic acute regulatory protein gene (StAR) through interactions with the transcription factors steroidogenic factor 1 (SF-1; also know as Ad4BP) and DAX-1 (Sugawara *et al,* 2001, *Endocrinology* 142: 3570-3577).

RIP140 is known to be essential for female fertility. Female mice null for RIP140 are viable but infertile because of complete failure to release oocytes at ovulation (White *et al*, 2000, *Nature Medicine*, 6:1368-1374). RIP140 is thought to have a secondary role in maintenance of pregnancy (Leonardsson *et al*, 2002, *Endocrinology*, 143(2): 700-707).

Surprisingly, it has now been discovered that RIP140 plays a role in controlling fat storage. Mice null for RIP140 are leaner than wild-type mice and fat in food-deprived RIP140 null mice appears to be deposited in muscle cells in greater amounts than in food-deprived wild-type mice. In addition, genes that are expressed only in brown adipose tissue in wild-type mice appear to be expressed in the white adipose tissue of mice null for RIP140. Unlike white adipose tissue, brown adipose tissue can be used as an energy source as a result of uncoupling and the leanness of mice null for RIP140 may result, in part, from an ability to use white adipose tissue as an energy source. It is also possible that the leanness of RIP140 null mice results from RIP140 having a role in adipocyte function which is inhibited in the null mice.

Because RIP140 null mice have decreased fat storage, compounds that modulate the activity of RIP140 may modulate fat storage. RIP140 is a co-regulator which exerts its effects by binding to nuclear receptor family members such as ER, RAR, TR, RXR, VDR or PPAR or transcription factors such as AhR, SF-1 and DAX-1. These proteins to which RIP140 specifically binds are referred to herein as target proteins. Compounds may modulate the activity of RIP140 by promoting or inhibiting its ability to form a complex with one or more of these target proteins. Compounds that modulate the ability of RIP140 to form a complex with one or more of these target proteins may act to decrease or increase fat storage.

Compounds that bind to RIP140 may promote or inhibit its ability to form a complex with a target protein. Similarly, compounds that bind to a target protein of RIP140 may promote or inhibit the ability of RIP140 to form a complex with that target protein.

As a first method, the invention therefore provides a method of screening for compounds that bind to RIP140, said method comprising assessing binding of a candidate compound to RIP140. The invention further provides a method of screening for compounds that bind to a target protein of RIP140, said method comprising assessing binding of a candidate compound to the target protein. The target protein of RIP140 is preferably selected from AhR, ER, RAR, TR, RXR, VDR, PPAR, SF-1 and DAX-1.

As a second method, the invention provides a method of screening for compounds that bind to a complex of RIP140 and a target protein, said method comprising assessing binding of a candidate compound to a complex of RIP140 and a target protein.

The invention further provides a third method of screening for compounds that modulate the formation or maintenance of a complex of RIP140 and a target protein. A modulator may be either an agonist or an antagnosist of the RIP140:target protein interaction. An agonist is a compound that promotes the formation and/or maintenance of a complex of RIP140 and a target protein. An antagonist is a compound that inhibits the formation and/or maintenance of a complex of RIP140 and a target protein.

Preferably, the method of screening for compounds that act to modulate the formation or maintenance of a complex of RIP140 and a target protein comprises the steps of:

(a) mixing RIP140, a target protein and one or more candidate compounds;

(b) incubating the mixture to allow RIP140, the target protein and the candidate compound(s) to interact; and

(c) assessing whether the interaction between RIP140 and the target protein is modulated.

The mixing of RIP140, the target protein and candidate compound in step (a) may be done in any order.

The candidate compounds used in the third method may be compounds already identified as binding to RIP140 or a target protein by the first method of the invention, or compounds identified as binding to a complex of RIP140 and a target protein by the second method of the invention.

***In vivo* confirmation of function of compounds identified**

Once a candidate compound has been identified *in vitro* as a compound that binds to RIP140 or to a target protein, as a compound that binds to a complex of RIP140 and a target protein, or as a modulator of the interaction between a target protein and RIP140, it may be desirable to perform further experiments to confirm the *in vivo* function of the compound in modulating fat storage. Any of the above methods may therefore comprise the further steps of administering to a mammal a candidate compound and assessing its effect on fat storage.

The invention also provides a method of assessing the *in vivo* effect on fat storage of a compound obtained or obtainable by any of the methods described above, comprising administering the compound to a mammal and assessing the effect on fat storage.

In general, therefore, the invention provides a method for screening for a compound that modulate fat storage in a mammal, comprising a first step of identifying a compound which: (a) binds to RIP140 or to a RIP140 target protein; (b) binds to a complex of RIP140 and a RIP140 target protein; or (c) modulates the binding interaction between RIP140 and a RIP140 target protein, and comprising a second step of administering to a mammal a candidate compound identified in step (a), (b) or (c) and assessing its effect on fat storage in the mammal.

The mammal may be any species of mammal including humans but is preferably a monkey, pig, rabbit, guinea pig, rat or mouse. Tests on non-humans may be preferred.

The compound identified by a method of the invention may be administered to animal models of obesity and leanness. The compound may be administered to an animal in which a gene or combination of genes known to be involved in fat storage has been knocked-out. For example, the compound may be administered to an animal that is null for RIP140 or an animal that is null for the *ob* gene. Other models are widely available (*e.g.* Pomp (1999) *Molecular Medicine Today* 5:459-460).

The methods of screening for compounds that bind to RIP140 or to a target protein, or that bind to a complex of RIP140 and a target protein, or that modulate the interaction between RIP140 and a target protein may take place in a different geographical location from the method for assessing the effect of the compounds identified on fat storage.

Direct screening for compounds that bind to RIP140 or to a target protein, or that bind to a complex of RIP140 and a target protein:

The RIP140 or target protein used in the method of screening for compounds that bind RIP140 or a target protein may be free in solution, affixed to a solid support, located on a cell surface or located intracellularly.

Preferably, the binding of a candidate compound to RIP140 or to a target protein is detected by means of a label directly or indirectly associated with the candidate compound. The label may be a fluorophore, radioisotope, or other detectable label.

For example, in a method of screening whether a candidate compound binds to RIP140, one or both of the candidate compound and RIP140 may be labelled with a fluorescent label such that the binding between the candidate compound and RIP140 may be detected by an intrinsic fluorescence change which occurs when the candidate compound binds to RIP140. For example, the candidate compound may be joined to a fluorescence resonance energy transfer (FRET) donor and RIP140 to a FRET acceptor (or *vice versa*) such that, when the candidate compound

and RIP140 interact, stimulation of the FRET donor excites the FRET acceptor causing it to emit photons. Interaction may be also be detected by fluorescent labelling of the candidate compound and/or RIP140 such that fluorescence is quenched when they form a complex.

Other methods for assessing interaction between the candidate compound and RIP140 or between a candidate compound and a target protein may include using NMR to determine whether a RIP140:candidate compound complex or a target protein:candidate compound complex is present.

The presence of a RIP140:candidate compound complex or a target protein:candidate compound complex may also be detected as a band at a particular position when run on a gel.

Another method of assessing interaction between RIP140 and a candidate compound may involve immobilising RIP140 on a solid surface and assaying for the presence of free candidate compound. If there is no interaction between the candidate compound and RIP140, free candidate compound will be detected. The candidate compound may be labelled to facilitate detection. This type of assay may also be carried with the candidate compound being immobilised on the solid surface. Interaction between the immobilised RIP140 and the free candidate compound may also be monitored by a process such as surface plasmon resonance.

Other methods for studying RIP140 interactions are described by Sugawara *et al,* 2001, *Endocrinology* 142: 3570-3577.

These techniques described above for use with RIP140 can, of course, be used *mutatis mutandis* for any of the target proteins of the invention *e.g.* to detect whether a candidate compound binds to a target protein.

The methods of screening for compounds that bind to a complex of RIP140 and a target protein can be carried out using the methods described above but using a complex of RIP140 and a target protein in place of RIP140 or in place of the target protein.

Direct screening for modulators of a complex of RIP140 and a target protein:

Modulation of the interaction between RIP140 and a target protein in the presence of candidate compounds may be assessed directly. Various methods for direct detection of protein/protein interactions are available.

The methods described above for assessing whether a candidate compound binds to RIP140, to a target protein, or to a complex of RIP140 and a target protein, can also be used to assess whether a candidate compound modulates the interaction between RIP140 and a target protein. The target

protein is already known to bind RIP140 and the methods described above are hence used to assess whether that interaction is disrupted or promoted by the candidate compound.

For example, one of both the target protein and RIP140 may be labelled with a fluorescent label so that interaction between the target protein and RIP140 may be detected by an intrinsic fluorescence change that occurs when the RIP140:target protein complex is formed or disrupted in the presence of a candidate compound.

Interaction of RIP140 and a target protein in the presence of candidate compound may also be assessed by detecting the accessibility of peptide sequences (*e.g.* epitopes) on RIP140 and/or the target protein that are masked when the two proteins form a complex. For example, motifs on RIP140 that interact with nuclear receptors have been identified in WO98/49561, Lee *et al* (Mol Cell Biol, 1998, 18(11): 6745-44) and in Wei *et al* (J. Biol. Chem., 2001, 276(19): 16107-12). A lack of interaction between RIP140 and a target protein that is a nuclear receptor in the presence of a candidate compound may therefore be determined by detection of such motifs, for example, using antibodies.

Indirect screening for modulators of RIP140:target protein complex formation and maintenance using two-hybrid systems:

Indirect methods for assessing whether the interaction between a target protein and RIP140 is modulated in the presence of a candidate compound may also be used. One indirect method of screening for modulation of the interaction between target protein and RIP140 in the presence of a candidate compound involves using a two-hybrid system. The target protein may be fused to an activation domain of a transcription factor and RIP140 to a DNA-binding domain of a transcription factor (or *vice versa*), such that interaction between the target protein and RIP140 promotes the transcription of a reporter gene in a cell.

The invention provides a method of screening for compounds that modulate the interaction between the target protein and RIP140, said method comprising:

a) contacting a cell containing a nucleic acid molecule comprising a promoter operatively linked to a reporter gene with: (i) a first fusion protein comprising one of the target protein and RIP140 fused to the activation domain of a transcription factor, (ii) a second fusion protein comprising the other of the target protein and RIP140 fused to the DNA-binding domain of a transcription factor; and (iii) a candidate compound; and

b) assessing the level of expression of the reporter gene,

wherein interaction between the target protein and RIP140 promotes transcription of the reporter gene by activating said promoter.

This method may be used to assess interaction between RIP140 and the target protein in any eukaryotic cell. Preferably, the method is used to assess the interaction between RIP140 and the target protein in a yeast cell or a mammalian cell. Where the candidate compound is an organic compound and a yeast two-hybrid system is being used, the permeability of the yeast cell wall is preferably enhanced *e.g.* by using chemicals such as polymyxin B.

The level of expression of a reporter gene in the two-hybrid system is indicative of the level of interaction between the target protein and RIP140. A candidate compound that inhibits the interaction between the target protein and RIP140 decreases or abolishes the level of expression of the reporter gene. A candidate compound that promotes the interaction between the target protein and RIP140 maintains or increases the level of expression of the reporter gene.

Preferably, the reporter gene is easily assayed. For example, the reporter gene may give a detectable signal, such as a visible signal. The reporter gene may encode a protein which gives a visible signal itself, or which catalyses a reaction which gives a visible signal or change *e.g.* a fluorescent protein or an enzyme. The reporter gene may encode an enzyme such as a beta-galactosidase or a peroxidase, both of which are commonly used with coloured substrates and/or products. The reporter gene may encode a green fluorescent protein (GFP) or a fluorescent derivative thereof such as YFP or CFP (see Prasher *et al*, 1995, *Trends Genet* 11(8): 320). The reporter gene may encode a luminescent protein, such as luciferase.

The reporter gene may drive DNA replication (Vasavada *et al*, 1991, *PNAS*, 88:10686-10690) in the cell or may encode a drug resistance marker (Fearon *et al*, 1992, *PNAS* 89: 7958-7962).

The reporter gene may encode a protein that enables positive selection of cells in which the interaction between the target cell and RIP140 is inhibited. For example, the reporter gene may encode a protein that is toxic or cytostatic so that only cells that do not express the reporter are able to survive or grow. As a result, the only cells to survive are those in which a candidate compound inhibits the interaction between the target protein and RIP140 so that the reporter gene is not expressed. Examples of reporter genes of this type that may be used in yeast include URA3, LYS2 and CYH2 (see Vidal *et al*, 1996, *PNAS*, 93: 10315-10320). The protein encoded by the reporter gene may also prevent cell growth in the absence or presence of a particular amino acid or other component in cell media. For example, the reporter gene may encode a DNA-binding protein, Tn10 tetracycline, which represses transcription of a TetRop-HIS3 gene

so that yeast cells in which the reporter gene is expressed do not grow in the absence of histidine (see Shih et al, 1996, *PNAS*, 93: 13896-13901). In contrast, yeast cells in which the interaction between RIP140 and the target protein has been disrupted do not express TN10 tetracycline and are consequently able to grow in the absence of histidine.

The proteins encoded by the reporter genes may be in the form of fusion proteins. Methods for the generation of fusion proteins are standard in the art and will be known to the skilled reader. For example, most general molecular biology, microbiology recombinant DNA technology and immunological techniques can be found in Sambrook *et al.*, (Molecular Cloning, A Laboratory Manual, Cold Harbor-Laboratory Press, Cold Spring Harbor, N.Y., 2000) or Ausubel *et al.*, (Current Protocols in Molecular Biology, Wiley Interscience, NY, 1991).

Other indirect screening methods for modulators of RIP140:target protein interaction:

Many of the target proteins of RIP140 are transcription factors which are activated or inhibited by interaction RIP140. For example, SF-1 is a transcription factor which is inhibited by RIP140 while transcription of genes by AhR is activated by RIP140 (Kumar *et al*, J. Biol. Chem. (1999) 274:22155-22164). Interaction between RIP140 and a target protein of this type may also be assessed indirectly by means of a reporter gene under the control of a promoter which is regulated by the target protein. Where binding of RIP140 to the target protein inhibits transcription of the reporter gene, disruption of the RIP140:target protein complex in the presence of a candidate compound will result in expression of the reporter gene. Conversely, where binding of RIP140 to the target protein promotes transcription of the reporter gene, disruption of the RIP140:target protein interaction will inhibit transcription of the reporter gene.

The invention provides a method of screening for compounds that modulate the interaction between RIP140 and a target protein which is a transcription factor, said method comprising:

a) contacting a nucleic acid molecule, comprising a target protein-regulated promoter operatively linked to a reporter gene, with one or more candidate compounds, in the presence of RIP140 and the target protein; and

b) assessing the level of expression of the reporter gene.

This method employs a nucleic acid molecule comprising a promoter operatively linked to a reporter gene, such that transcription of the reporter gene is under the control of the promoter and is regualted by the target protein. These nucleic acids are referred to as reporter constructs.

The promoter in the construct is a target protein-regulated promoter from which the target protein is able to promote transcription of the reporter gene. The nature of the promoter will depend on the identity of the target protein. Where the target protein is a nuclear hormone

receptor, such as ER or TR, the promoter contains a hormone response element to which the nuclear hormone receptor binds to initiate transcription. SF-1 regulated promoters contain at least one gonadotroph specific element (GSE) to which SF-1 binds to initiate transcription (Bryan et al, 1999, *J. Molec. Endocrin*, 22:241-249).

The target protein-regulated promoter may be derived from the region upstream of any gene whose transcription is regulated by the target protein.

The reporter gene controlled by the promoter may be a gene which is regulated by the target protein in nature. In such situations, the reporter construct preferably includes the reporter gene and its natural upstream regulatory sequences. Preferably, however, the promoter controls transcription of a heterologous reporter gene which is easily assayed, as described above for two-hybrid methods. However, in the two-hybrid system, compounds that inhibit the interaction between that target protein and RIP140 are always detected as a result of a decrease in the expression of the reporter gene. Where the transcriptional activity of the target protein is itself detected, the result depend on whether RIP140 acts to activate or inhibit the target protein's activity: compounds that inhibit the interaction between RIP140 and the target protein give an *increase* in the expression of the reporter gene when RIP140 *inhibits* the activity of the target protein, but give a *decrease* in expression of the reporter gene when RIP140 *activates* the activity of the target protein.

For example, where the reporter gene is a fluorescent protein, inhibition of the interaction between RIP140 and a target protein that is *inhibited* by RIP140 may be detected by an *increase* in fluorescent protein expression (*cf.* two-hybrid system). In contrast, inhibition of the interaction between RIP140 and a target protein that is *activated* by RIP140 may be detected by a *decrease* in fluorescent protein expression. Similarly, where the reporter gene is a toxic gene, inhibition of the interaction between RIP140 and a target protein that is *inhibited* by RIP140 may be detected by an increase in cell *death*. In contrast, inhibition of the interaction between RIP140 and a target protein that is *activated* by RIP140 may be detected by an increase in cell *survival*.

The proteins encoded by the reporter genes may be in the form of fusion proteins as described above. For example, genes that give a visible signal may be fused downstream of a gene that is linked in nature to the promoter in the construct.

Vectors comprising reporter constructs:

Reporter constructs used in the indirect screening methods of the invention may be in the form of a viral vector or a non-viral vector. Preferably, the nucleic acid molecules used in these methods

of the invention are in the form of a conventional non-viral vector, such as a plasmid. Where these indirect screening methods are conducted in cell-based or tissue-based assays, the introduction of the non-viral vector into the animal cells may be carried out by any method known in the art including dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of polynucleotides in liposomes or direct microinjection of the DNA into nuclei, *etc.*

Use of nucleic acid molecules:

The invention provides the use of a reporter construct, as described above, in a method of screening for compounds that modulate fat storage.

Systems for carrying out screening methods

The methods of the invention may be carried out in cell-free systems or in cells or tissues.

In particular, the indirect screening methods described above may be carried out in a cell-free system, in a cell or in a tissue. The cell-free system must contain all the necessary components for transcription of the reporter gene where the level of expression is detected by measuring mRNA levels, and all the necessary components for transcription and translation of the reporter gene where the level of expression is assessed by measuring protein levels.

It is preferred that the methods of screening of the invention be conducted in cell-free systems since this facilitates high-throughput screening of candidate compounds.

Indirect screening methods of the invention are preferably carried out in eukaryotic cells, such as mammalian (*e.g.* human) cells or tissues, or yeast cells.

When the indirect method of screening for compounds that inhibit the interaction of RIP140 and a target protein using a nucleic acid molecule comprising a target protein-regulated promoter is carried out in a cell, the cell should preferably express both RIP140 and the target protein endogenously. If RIP140 and the target protein are not endogenously expressed, they may be introduced into the cell using a viral or non-viral vector encoding the RIP140 or target protein. Preferably, RIP140 and the target protein are introduced into the cell in the form of plasmids.

Assessing level of expression of reporter gene:

The level of expression of a reporter gene may be assessed by measuring the level of a mRNA transcribed from the reporter gene or the level of protein translated after its transcription. The measurement methods may be qualitative or quantitative.

Measuring level of mRNA

The level of mRNA transcribed from a reporter gene can be assessed, for example, by traditional blotting techniques described in Sambrook *et al* [*supra*]. Messenger RNA can be purified and separated using gel electrophoresis. The nucleic acids on the gel are then blotted onto a solid support, such as nitrocellulose. The solid support is exposed to a labelled probe and then washed to remove any unhybridized probe. Next, the duplexes containing the labelled probe are detected. Typically, the probe is labelled with a radioactive moiety.

Alternatively, the level of mRNA transcribed from the reporter gene may be detected by PCR-based methods. The mRNA transcribed from the reporter gene may be specifically amplified using primers that only bind to the mRNA with the amplified mRNA being detected using the blotting methods described above. The level of transcription of the reporter gene could be also detected using fluorescence resonance energy transfer (FRET) through fluorophores coupled to two oligonucleotides that are complementary to the mRNA transcribed from the reporter gene. (see Wouters *et al*, 2001, *Trends in Cell Biology* 11, 203-211).

As the cell-free system, cell or tissue will contain DNA from which the mRNA is transcribed, it is preferred to use a RNA-specific detection technique or to focus on sequence present in the mRNA transcription but not in the DNA (*e.g.* splice junctions, polyA tail *etc.*). The methods of the invention may comprise an initial step of: extracting mRNA from the cell-free system, cell or tissue; removing DNA from the cell-free system, cell or tissue; and/or disrupting DNA but not mRNA in the cell-free system, cell or tissue.

Methods for selectively extracting RNA from biological samples are well known and include methods based on guanidinium buffers, lithium chloride, acid phenol:chloroform extraction, SDS/potassium acetate, *etc.* After total RNA has been extracted, mRNA may be enriched for example using oligo-dT techniques. Methods for removing DNA from biological samples include DNase digestion. Methods for removing DNA encoding the reporter gene but not the RNA transcribed from it will use an agent which is specific to a sequence within the DNA.

Measuring level of protein

Measurement of mRNA levels is not ideal in high throughput screening methods, so it is preferred that reporter gene expression is assessed by measuring protein levels.

The level of protein expressed from the reporter gene can be conveniently measured by using an antibody which binds to the protein encoded by the reporter gene. Following removal of unbound antibody, the level of the protein encoded by the reporter gene can be determined by

assessing the level of the antibody bound to it. This may be done by labelling the antibody that binds to the protein or by using a second labelled antibody which binds to the first antibody.

Where the reporter gene encodes a protein that provides a visible signal, the level of expression of the reporter gene is preferably assessed by detecting the visible signal. For example, where the reporter gene encodes a fluorescent protein such as GFP, or an enzyme such as luciferase, the level of expression may be assessed by fluorescence/luminescence detection. Where the reporter gene encodes a protein that is toxic or cytostatic, the level of expression may be assessed by looking at cell survival or cell growth.

Reference standards:

A reference standard (*e.g.* a control), is typically needed in order to detect whether the interaction between the target protein and RIP140 is modulated in the method of the invention. In order to detect whether a candidate compound inhibits the interaction between the target protein and RIP140, the interaction between target protein and RIP140 in the presence of a candidate compound may be compared with the interaction between the target protein and RIP140 in the absence of a candidate compound.

The reference may have been determined before performing the method of the invention, or may be determined during (*e.g.* in parallel) or after the method has been performed. It may be an absolute standard derived from previous work.

Proteins for use in methods of the invention

The methods of the invention may use target proteins and RIP140 derived from any eukaryote. Preferably, they use target proteins and RIP140 derived from an animal, such as a mammal. Preferably, RIP140 and the target protein used in a method of the invention are both derived from the same mammal. The RIP140 and target protein are preferably both human proteins. The RIP140 gene has been cloned in a number of mammalian species including humans (Cavailles *et al,* 1995, *EMBO J.*, 14:3741-3751) and mouse (Lee *et al,* 1998, *Mol Cell Biol,* 18: 6745-55), and variants including natural biological variants, allelic variants and mutants containing amino acid substitutions, insertions or deletions from the wild-type sequence are in the NCBI database.

References in the methods of the invention to the use of RIP140 include these and other variants, provided that, where the method involves a RIP140/target protein interaction, the variants should retain the ability to interact with the target protein of interest. Similarly, variants of target proteins may be used if they retain the ability to interact with RIP140.

For example, if a protein has a modular structure then the methods of the invention may focus on single modules within the protein, and in particular on modules which possess a particular binding activity (*e.g.* on the region of ER which is involved in the ER/RIP140 interaction). Motifs in RIP140 that interact with nuclear receptors are disclosed in WO98/49561, Lee *et al* (*Mol Cell Biol*, 1998, 18(11): 6745-44) and Wei *et al* (*J. Biol. Chem.*, 2001, 276(19): 16107-12). Thus fragments of target proteins that interact with RIP140 and fragments of RIP140 that interact with target proteins may used in the methods of the invention. Other suitable variants of RIP140 and target proteins are known from the literature.

Polypeptides that are structurally similar to target proteins and RIP140, or to fragments of RIP140 and target proteins that retain the ability to interact, may also be used in the methods of the invention. These may be derived from natural target proteins or RIP140 or they may be prepared synthetically or using techniques of genetic engineering. In particular, synthetic molecules that are designed to mimic the tertiary structure of target proteins or RIP140 and in particular the domains of the target protein and RIP140 that interact may be used in the methods of the invention. References to the use of target proteins and RIP140 in the methods of the invention include the use of polypeptides that are structurally similar to target proteins and RIP140, or to fragments thereof.

References to the use of target proteins and RIP140 in the methods of the invention also include the use of fusion proteins comprising target proteins or RIP140, fusion proteins comprising variants or fragments thereof, or fusion proteins comprising polypeptides that are structurally similar to target proteins or RIP140 or to fragments of target proteins or RIP140. Such fusion proteins are particularly useful in two-hybrid methods.

Candidate compounds

Candidate compounds used in screening methods:

Typical candidate compounds for use in all the screening methods of the invention include, but are not restricted to, peptides, peptoids, proteins, lipids, metals, small organic molecules, RNA aptamers, antibiotics and other known pharmaceuticals, polyamines, antibodies or antibody derivatives (*e.g.* antigen-binding fragments, single chain antibodies including scFvs, *etc*), and combinations or derivatives thereof. Small organic molecules have a molecular weight of about more than 50 and less than about 2,500 daltons, and most preferably between about 300 and about 800 daltons. Candidate compounds may be derived from large libraries of synthetic or natural compounds. For instance, synthetic compound libraries are commercially available from

MayBridge Chemical Co. (Revillet, Cornwall, UK) or Aldrich (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts may be used. Additionally, candidate compounds may be synthetically produced using combinatorial chemistry either as individual compounds or as mixtures.

It has been suggested that the binding of RIP140 to nuclear receptors may be inhibited by acetylation of the domains in RIP140 that interacts with the receptor (Vo *et al*, 2001, *Mol Cell Biol*, 21(18): 6181-8). Candidate compounds may therefore be compounds identified as acetylating RIP140 in a pre-screen.

Compounds identified by screening methods

The invention further provides a compound that binds to RIP140 or to a target protein, or that binds to a complex of RIP140 and a target protein, or that modulates the interaction between RIP140 and a target protein, obtained or obtainable by any of the methods described above. Preferably, the compounds of the invention are organic compounds.

There is also provided a composition comprising a compound binds to RIP140 or to a target protein, or that binds to a complex of RIP140 and a target protein, or that modulates the interaction between RIP140 and a target protein, obtained or obtainable by any of the methods described above.

Compounds that are found to modulate fat storage may be useful for the treatment of disorders associated with deviation in fat storage in their own right or may be lead compounds for the development of new drugs for the treatment of such disorders. They may also be useful in research into the regulation of fat storage.

Pharmaceutical uses of compounds identified

Once a compound has been identified using one of the methods of the invention, it may be necessary to conduct further work on its pharmaceutical properties. For example, it may be necessary to alter the compound to improve its pharmacokinetic properties or bioavailability. The invention extends to any compounds identified by the methods of the invention which have been altered to improve their pharmacokinetic properties, and to composition comprising those compounds.

The invention further provides compounds obtained or obtainable using the methods of the invention, and compositions comprising those compounds, for use as a medicament *e.g.* for increasing or decreasing fat storage. The invention also provides the use of compounds obtained or obtainable using the methods of the invention, or compositions comprising those compounds

in the manufacture of a medicament to increase or decrease fat storage. The compounds may be used to treat or prevent disorders associated with increased or decreased fat storage, such as obesity or anorexia. A method of increasing or decreasing fat storage comprising administering a compound obtained or obtainable by any one of the methods of the invention, or a composition comprising such a compound, to a mammal, preferably a human, is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Reduction in body fat and weakening of the peritoneal wall in RIP140 null mice.

Figure 2: A) Weight gain and adipose tissue deposition in wild-type (WT) and RIP140 knockout (KO) mice; B) MRI scans and whole body spectra of wild-type and RIP140 knockout mice.

Figure 3: Food intake is normal in RIP140 null mice.

Figure 4: Comparison of glucose tolerance tests in wild-type and RIP140 null (Nrip1 null) mice.

Figure 5: Comparison of white adipose tissue (WAT) histology and cell size in wild-type (WT) and RIP140 knockout (KO) mice.

Figure 6: Comparison of brown adipose tissue (BAT) and skeletal muscle histology in wild-type (WT) and RIP140 knockout (KO).

Figure 7: A) Comparison of differentiation to adipocytes in wild-type (WT) and RIP140 knockout (KO) cells; B) Expression analysis of RIP140 mRNA.

Figure 8: Relative expression of RIP140 in different tissues of a wild-type mouse.

Figure 9: Comparison of expression of specific genes in white adipose tissue (WAT) and liver of wild-type and RIP140 null mice on different diets (st=starved; no=normal; hf=high fat). Figure 9A compares expression of PPARg, C/EBPa, C/EBPb and SREBP. Figure 9B compares expression of Glut4, aP2, leptin and adipolectin. Figure 9C compares expression of G6Pase, PEPCK and 24p3. Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure10: Expression of C/EBPá in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO). Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 11: Expression of PPARã in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO). Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 12: Expression of SREBP1c in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO). Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 13: Expression of Glut4 in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO). Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 14: Expression of leptin in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO). Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 15: Expression of CPT1b in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO). Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 16: Expression of UCP1 in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO). Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 17: Expression of PPARá in white adipose tissue (WAT), brown adipose tissue (BAT), muscle and liver of faster and fed wild-type mice (WT) and RIP140 knockout mice (KO). Expression in wild-type mice is shown in left-hand column of each pair of columns and expression in RIP140 null mice in the right hand column of each pair of columns.

Figure 18: Comparison of triglyceride accumulation in liver tissue of RIP140 null mice compared to wild type when maintained on normal (a) or high fat (35%) diet (b) for 10 days.

EXAMPLES:

Various aspects and embodiments of the present invention will now be described in some detail. It will be appreciated that modification of detail may be made without departing from the spirit and scope of the invention.

RIP140 knockout (Nrip1 knockout) mice were found to be viable but to have impaired growth, as demonstrated by an approximately 20-25% reduction in adult body weight. This reduction is primarily a consequence of considerably less accumulation of total body fat (approx. 75%

reduction *vs.* wild-type), manifested in almost negligible appearance of subcutaneous fat and about half the amount of inguinal fat depots (Figures 1 and 2).

Food intake was found to be similar in RIP140 knockout mice and wild-type mice, suggesting that the fat reduction in the RIP140 knockout mice was not due to a difference in food intake (Figure 3). Glucose tolerance in the RIP140 knockout mice was similar to glucose tolerance in wild-type mice (Figure 4)

Histological analysis of fat tissue showed that cells derived from both white adipose tissue (WAT) and brown adipose tissue (BAT) are significantly smaller in the RIP140 knockout mice (Figures 5 and 6). Despite this defect, cultured cells isolated from WAT or mouse embryos (MEFs) of RIP140 knockout mice were able to differentiate to the same degree as wild-type cells in *in vitro* differentiation assays (Figure 7A). This suggests that differentiation *per se* is not impaired in RIP140 knockout mice and that leanness may result from impairment of the function of mature adipocytes. A functional role for RIP140 in mature adipocytes is supported by a dramatic up-regulation of RIP140 expression during differentiation of 3T3-L1 and MEF cells *vitro* (Soukas *et al,* 2001, J. Biol Chem., 36: 34167-34174) and the fact that WAT display the highest expression level of all mouse tissues examined (Figure 7B and Figure 8). However, the results of experiments carried out *in vitro* do not necessarily reflect the situation *in vivo* and it remains possible that lack of adipocyte differentiation is a contributing factor to the leanness observed in RIP140 knockout mice.

Detailed gene expression profile of mouse tissues revealed that the expression of a variety of genes was altered in the RIP140 null mice (Figure 9). Expression of key regulators of WAT function such as C/ EBPá (Figure 10), PPARã (Figure 11) and SREBPc (Figure 12) are all down- regulated (2- 10 times) in RIP140 knockout mice. As expected, this also leads to a decrease in target genes including Glut-4 (Figure 13) and Leptin (Figure 14).

In contrast, genes coding for two rate-limiting enzymes involved in fatty acid oxidation (CPT1b) and uncoupling protein (UCP-1) were both dramatically up-regulated (20-100 times) in RIP140 knockout WAT (Figures 15 and 16).

Since CPT1b and UCP- 1 are normally expressed at high levels in BAT, it is postulated that the WAT in RIP140 knockout mice may have adopted "BAT-like" function(s) which have led to increased fuel consumption (oxidation and uncoupling) and decreased storage in WAT of RIP140 knockout mice.

PPARá expression was also found to be up-regulated in RIP140 knockout mice (Figure 17).

The reduction of body fat in RIP140 knockout mice appears to be due to reduced adipose cell size. Expression analysis and *in vitro* differentiation suggest that the reduced adipose cell size may be due to impaired WAT function rather than impaired adipocyte differentiation. Possible mechanism(s) for the observed phenotype is an increased fatty acid transport (up-regulation of CTP1b) and/or increased uncoupling (up-regulation of UCP- 1) in mitochondria of WAT.

Additional possible phenotypes that may affect metabolism in RIP40 knockout mice are triglyceride accumulation in muscle and protection from fat accumulation in liver after high fat feeding (Figure 18).

CLAIMS

1. A method for screening for a compound that modulate fat storage in a mammal, comprising one of the following three steps:

   (i) identifying a compound which binds to RIP140 or to a RIP140 target protein;

   (ii) identifying a compound which binds to a complex of RIP140 and a RIP140 target protein; or

   (iii) identifying a compound which modulates the binding interaction between RIP140 and a RIP140 target protein,

   and further comprising the step of administering to a mammal a candidate compound identified in step (i), (ii) or (iii) and assessing its effect on fat storage in the mammal.

2. A method according to claim 1, wherein step (iii) comprises:
   a) mixing RIP140, the target protein and one or more candidate compounds;
   b) incubating the mixture to allow RIP140, the target protein and the candidate compound(s) to interact; and
   c) assessing whether interaction between RIP140 and the target protein is modulated.

3. A method according to claim 2 comprising:
   a) contacting a cell containing a nucleic acid molecule comprising a promoter operatively linked to a reporter gene with: (i) a first fusion protein comprising one of RIP140 and a target protein fused to the activation domain of a transcription factor, (ii) a second fusion protein comprising the other of RIP140 and a target protein fused to the DNA-binding domain of a transcription factor; and (iii) a candidate compound; and
   b) assessing the level of expression of the reporter gene,

   wherein interaction between RIP140 and the target protein promotes transcription of the reporter gene by activating said promoter.

4. A method according to claim 1 wherein step (iii) comprises:
   a) contacting a nucleic acid molecule, comprising a target protein-regulated promoter operatively linked to a reporter gene, with one or more candidate compound(s) in the presence of said target protein and RIP140; and
   b) assessing the level of expression of the reporter gene.

5. A method according to any one of claims 2 to 4, wherein the promoter controls transcription of a reporter gene with which it is linked in nature.

6. A method according to any one of claims 2 to 5, wherein expression of the reporter gene gives a detectable signal.

7. A method according to claim 6, wherein the reporter gene encodes a fluorescent protein, an enzyme, a toxic protein or cystostatic protein.

8. A method according to any preceding claim, wherein said method is carried out in a cell free system, a cell or a tissue.

9. A method according to any one of claims 2 to 8, wherein the nucleic acid molecule is in the form of a non-viral vector.

10. A method according to any one of claims 2 to 9, wherein the step of assessing the level of expression of the reporter gene comprises measuring the level of mRNA transcribed from the reporter gene.

11. A method according to any one of claims 2 to 9, wherein the step of assessing the level of expression of the reporter gene comprises measuring the level of protein translated after transcription of the reporter gene.

12. A compound that binds to RIP140 or to a target protein, obtained or obtainable by a method of claim 1.

13. A compound that binds to a complex of RIP140 and a target protein, obtained or obtainable by a method of claim 1.

14. A compound that modulates the interaction between RIP140 and a target protein, obtained or obtainable by a method of any preceding claim.

15. A method according to any one of claims 1 to 11, or a compound according to any one of claims 12 to 14, wherein the target protein is selected from AhR, ER, RAR, TR, RXR, VDR, PPAR, SF-1 and DAX-1.

16. A method of assessing the effect of a compound according to any one of claims 12 to 14 on fat storage, comprising administering the compound to a mammal and assessing its effect on fat storage.

17. A pharmaceutical composition comprising a compound according to any one of claims 12 to 14.

18. A compound according to any one of claims 12 to 14 for use as a medicament.

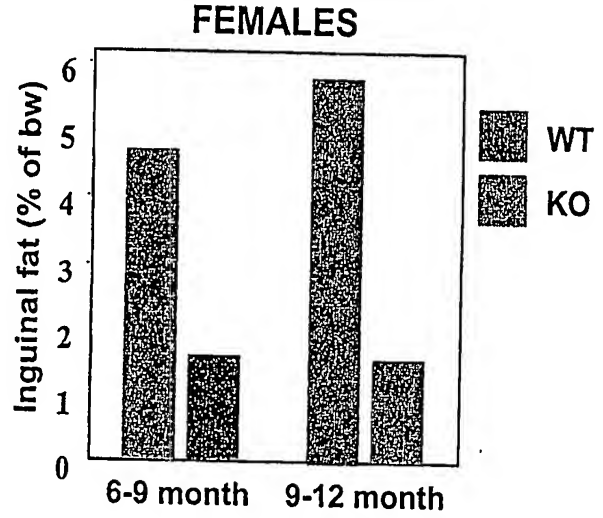
19. Use of a compound according to any one of claims 12 to 14 in the manufacture of a medicament for treating or preventing a disorder associated with increased or decreased fat storage.

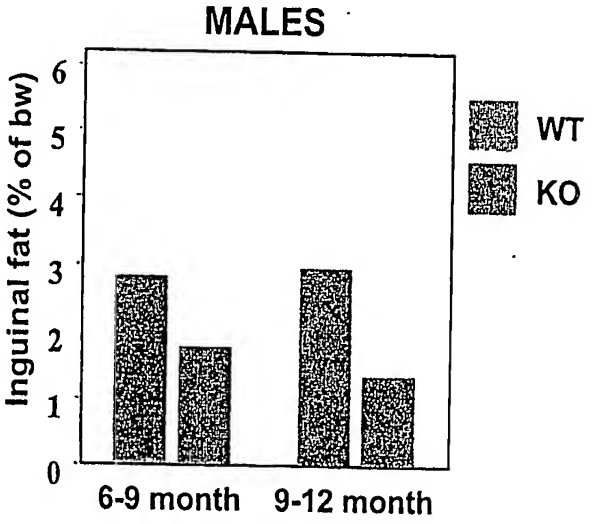
20. Use according to claim 19 wherein said disorder is obesity or anorexia.

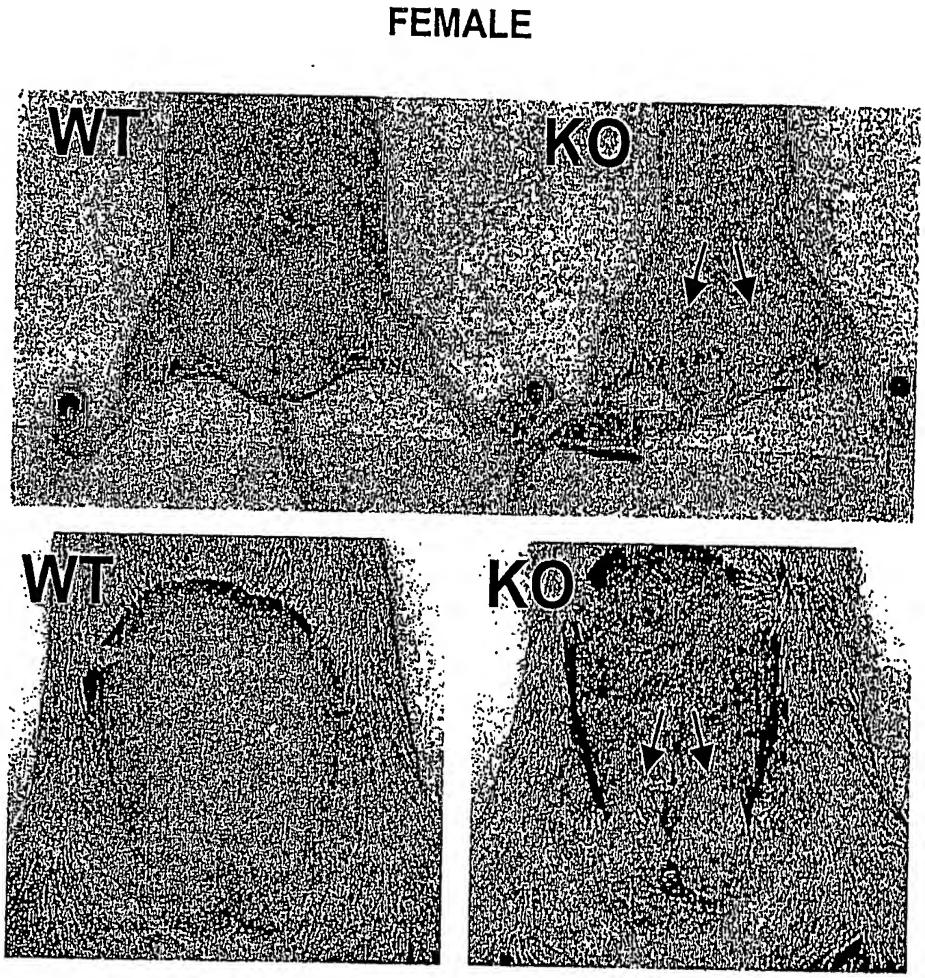
21. A method of altering fat storage in a mammal, comprising administering a compound according to any one of claims 12 to 14, or a composition according to claim 17.

F 1

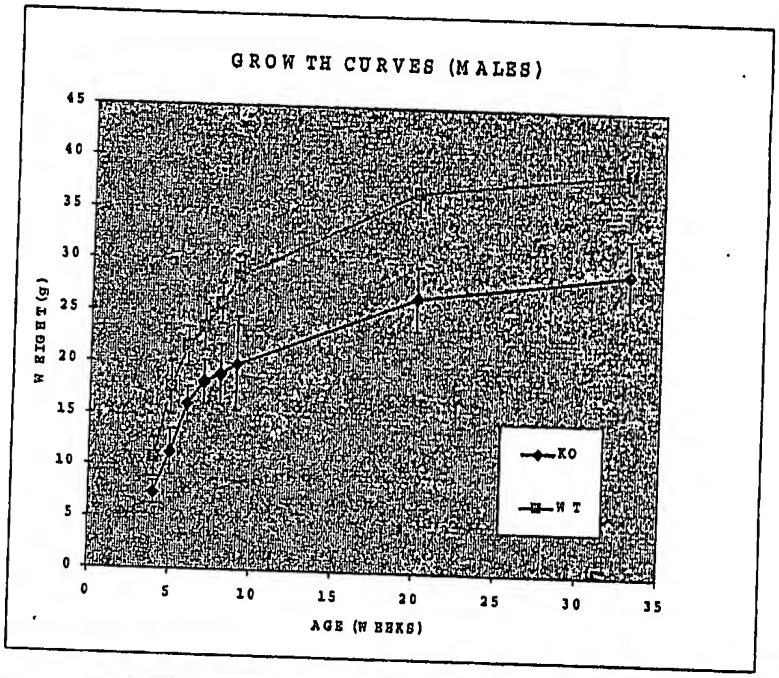
Reduction in body fat and weakening of the peritoneal wall (hernia) in RIPKO mice.



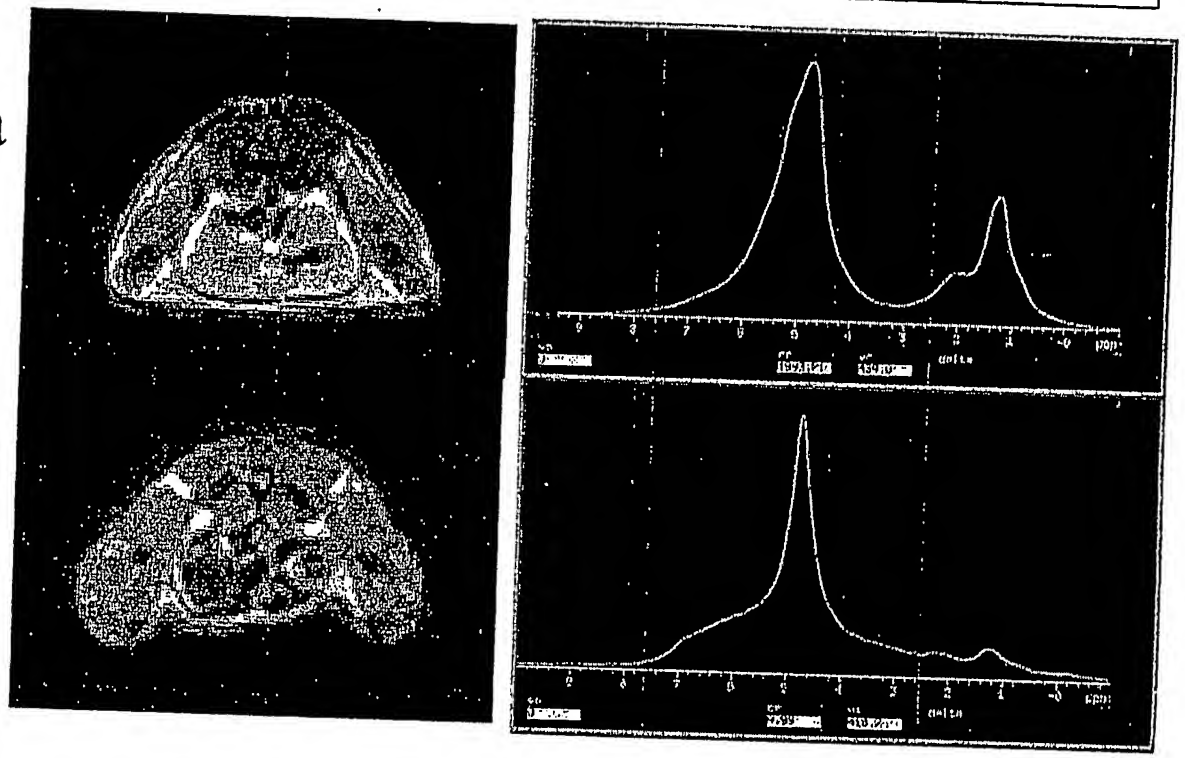




A) Weight gain and adipose tissue deposition in Nrip1 null mice



B) MRI scans and whole body spectra



WATER FAT

FIG

Food intake is normal in Nrip1 KO mice

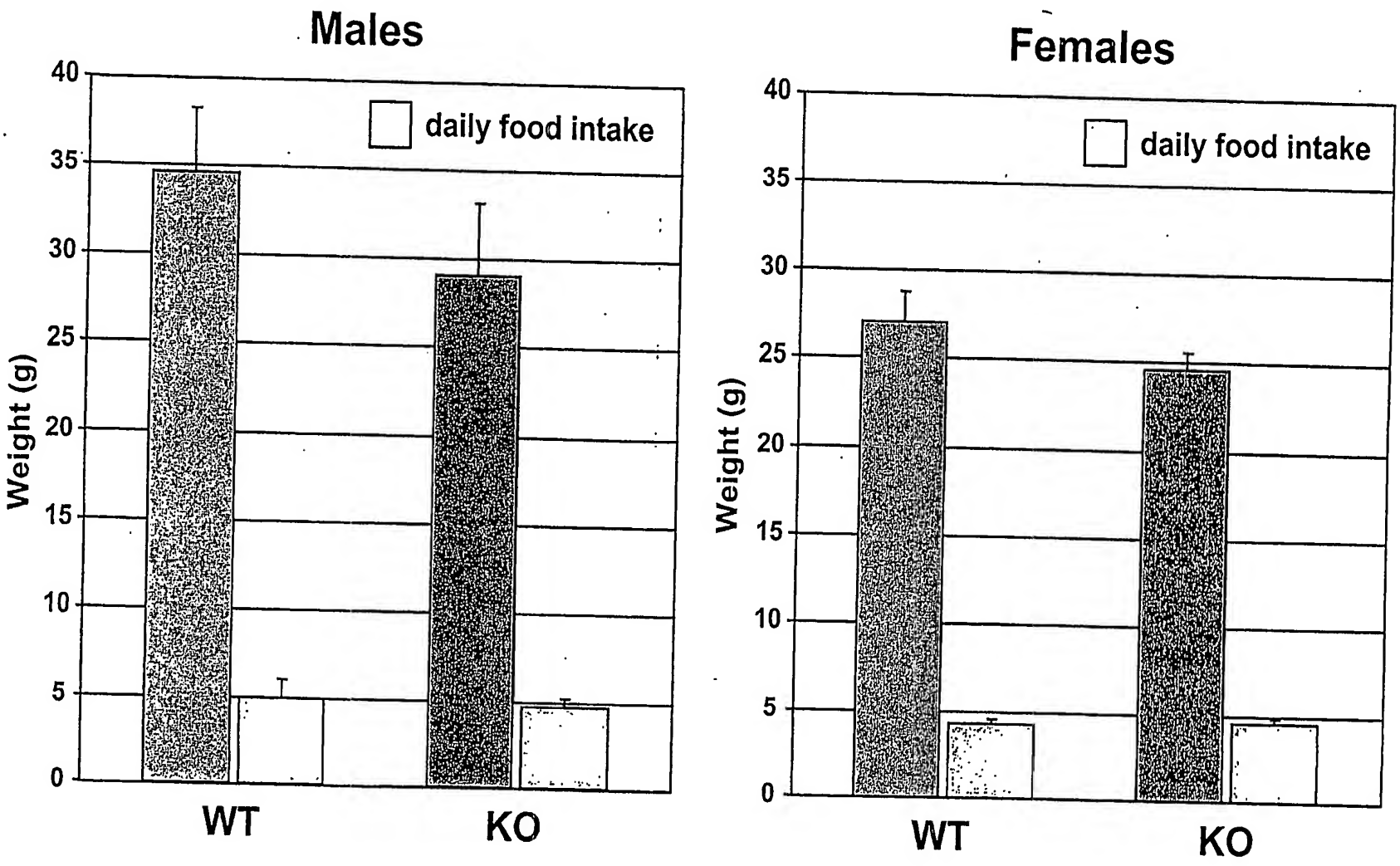
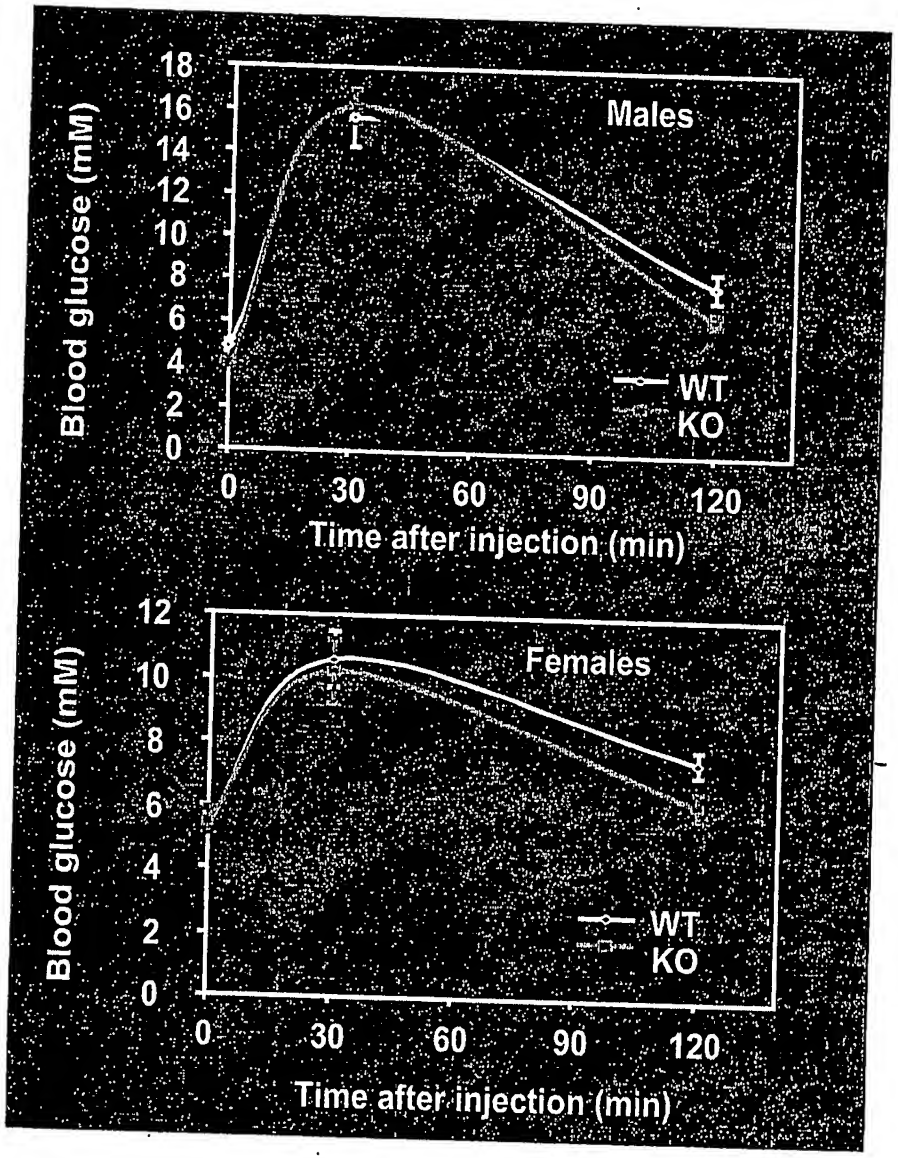
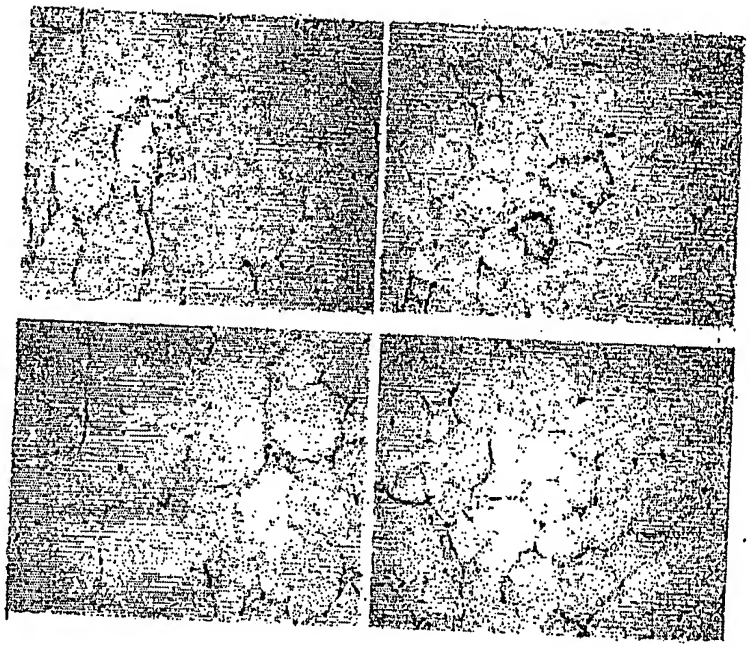


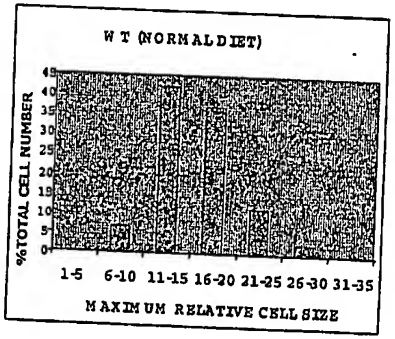
FIG 4

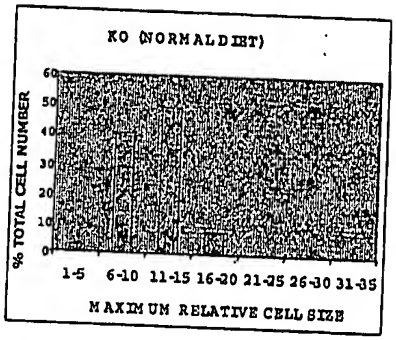
Comparison of glucose tolerance tests in wild type and Nrip1 null mice

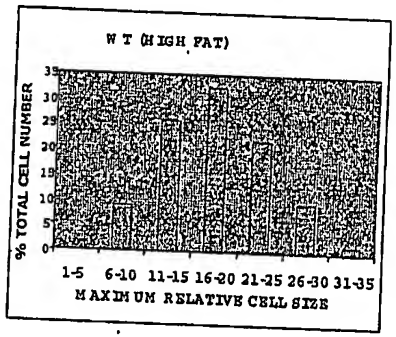


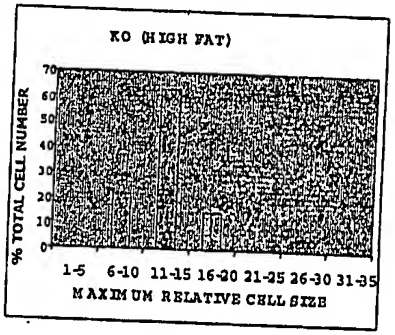
WAT histology and cell size





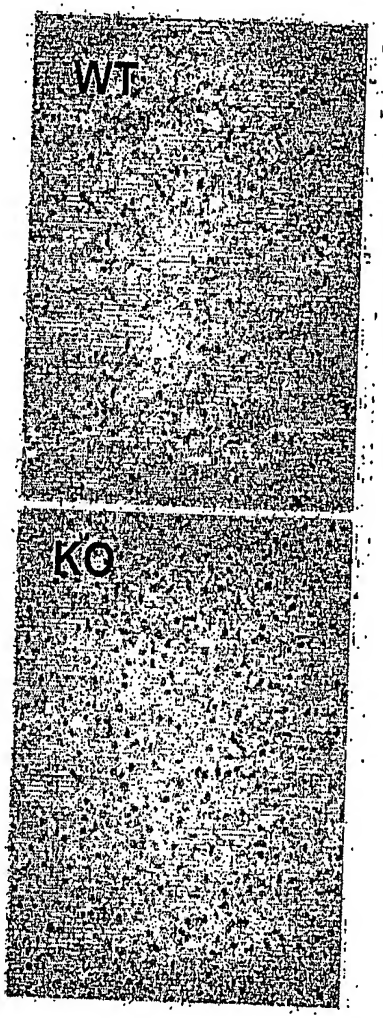






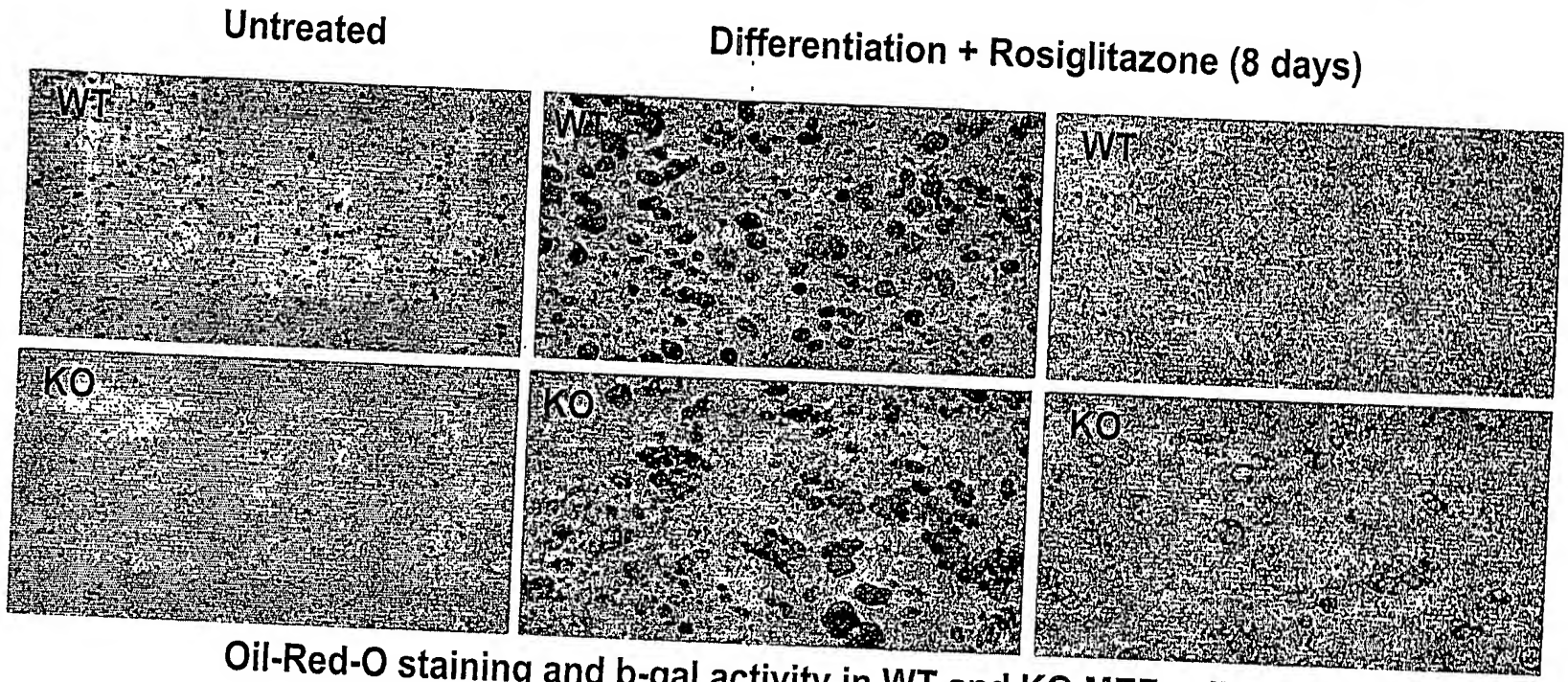
FIG

Histology of BAT from mice maintained for 10 days on a 35% (HIGH) fat diet.



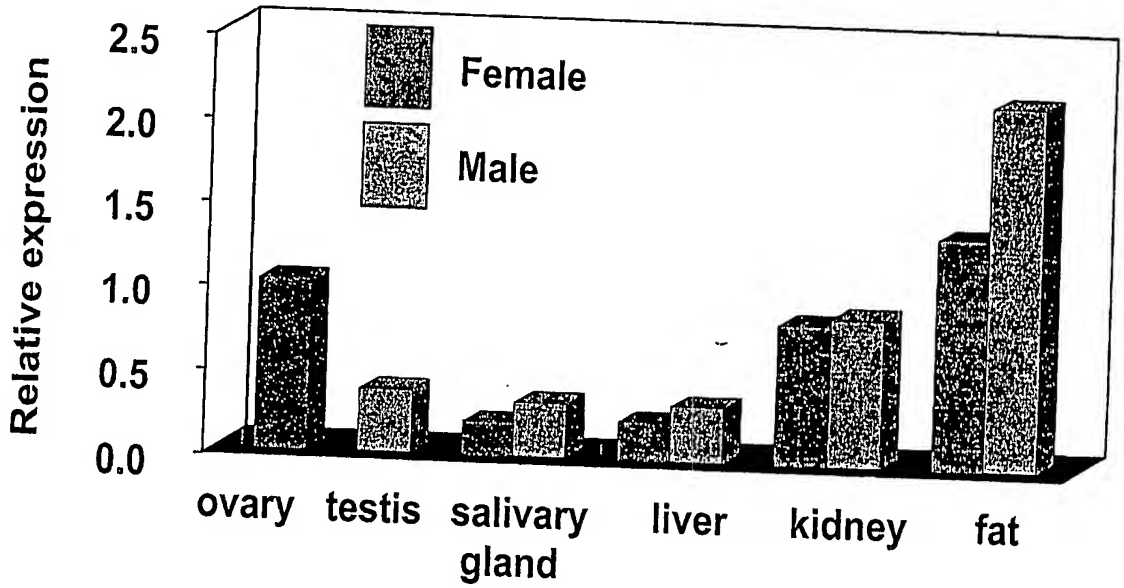
Histology of skeletal muscle from mice fasted for 18 hours

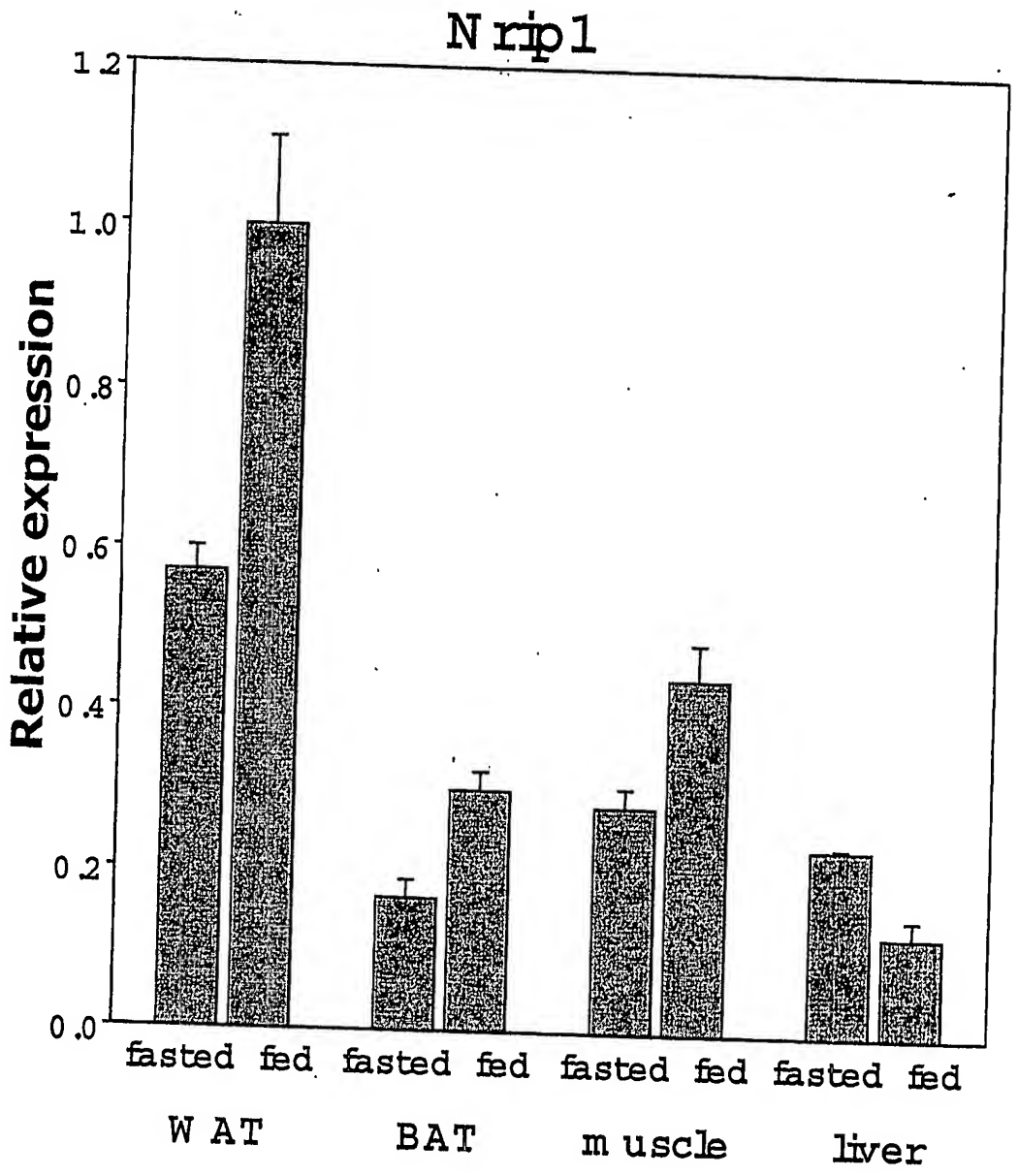
A) Nrip1/RIP140 null cell differentiation to adipocytes in vitro is unaffected

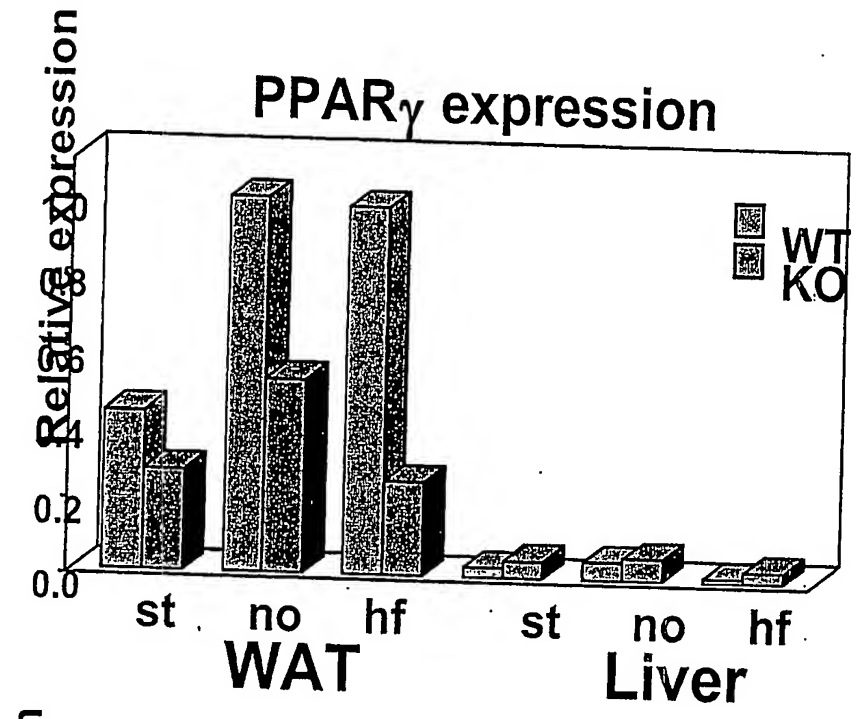


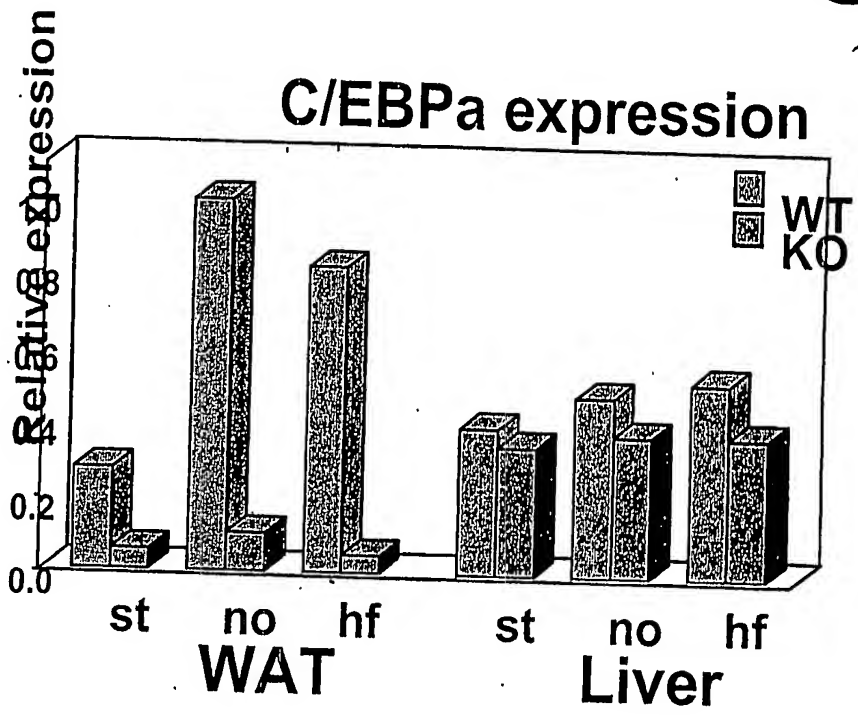
Oil-Red-O staining and b-gal activity in WT and KO MEF cells

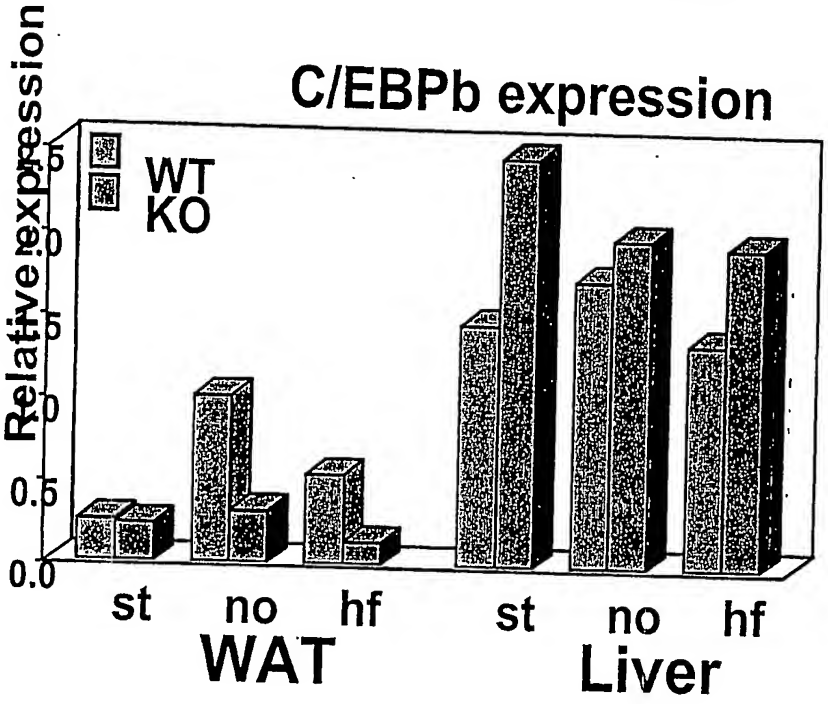
B) Expression analysis of Nrip1 mRNA

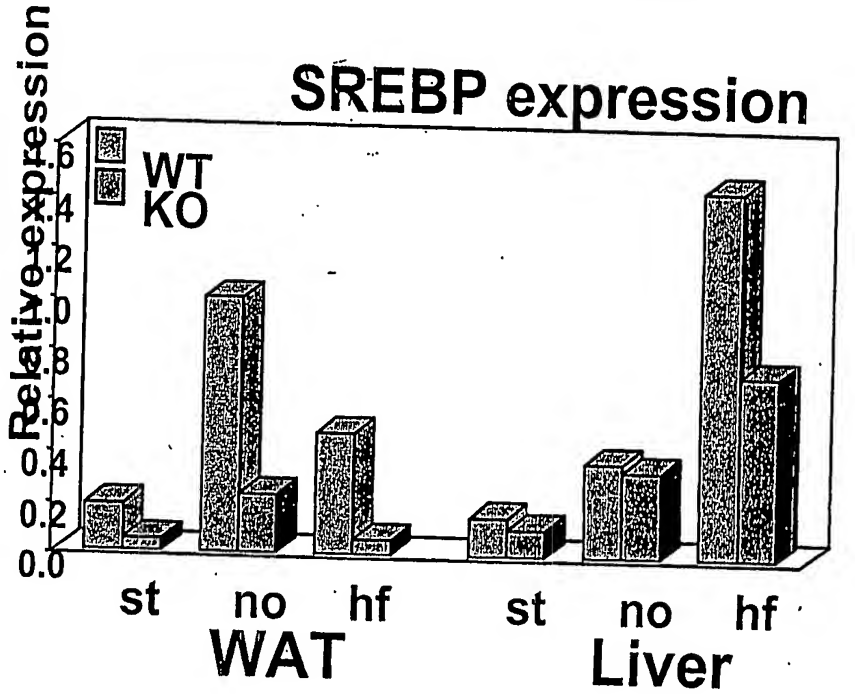


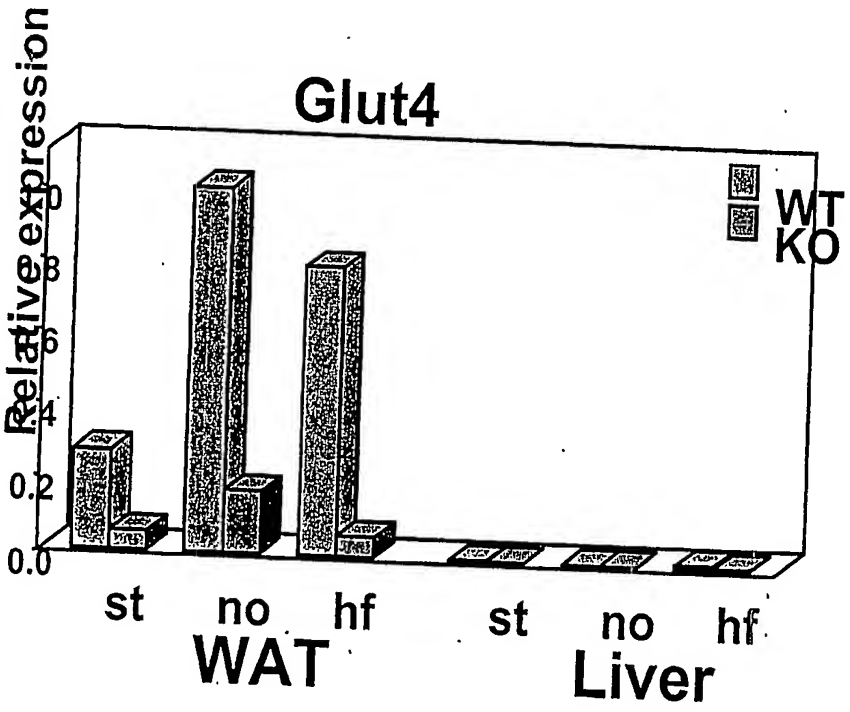

Nrip1
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
fasted
fed
fasted
fed
fasted
fed
fasted
fed
WAT
BAT
muscle
liver

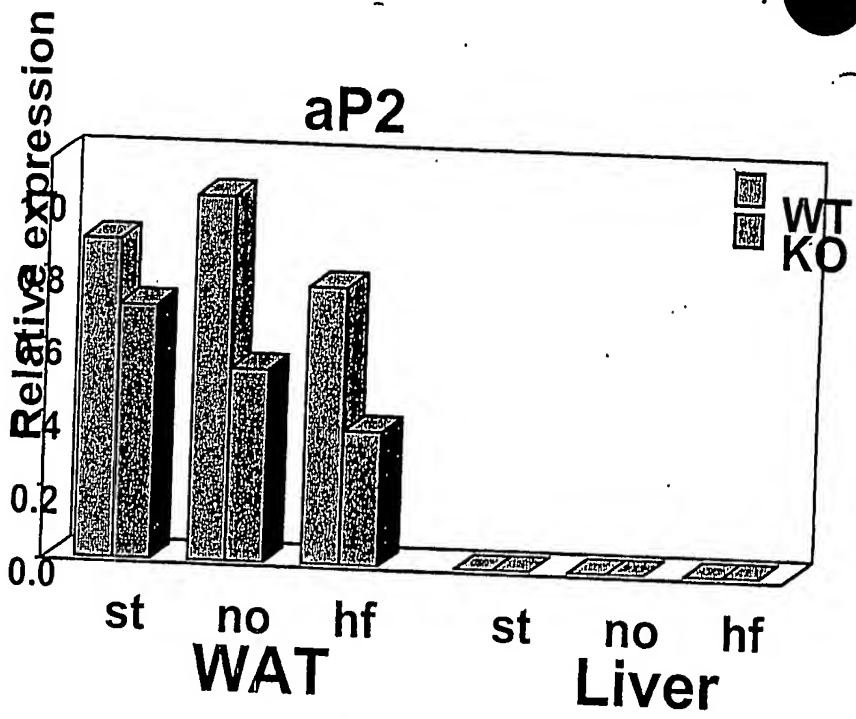

PPARγ expression
Relative expression
WT
KO
0.0
0.2
st
no
hf
st
no
hf
WAT
Liver

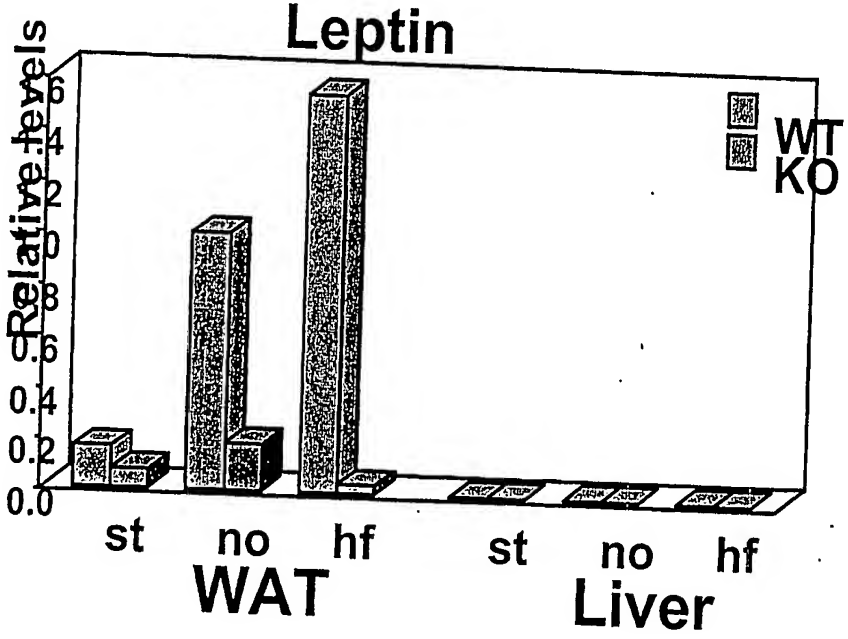

C/EBPa expression
Relative expression
WT
KO
0.0
0.2
st
no
hf
st
no
hf
WAT
Liver

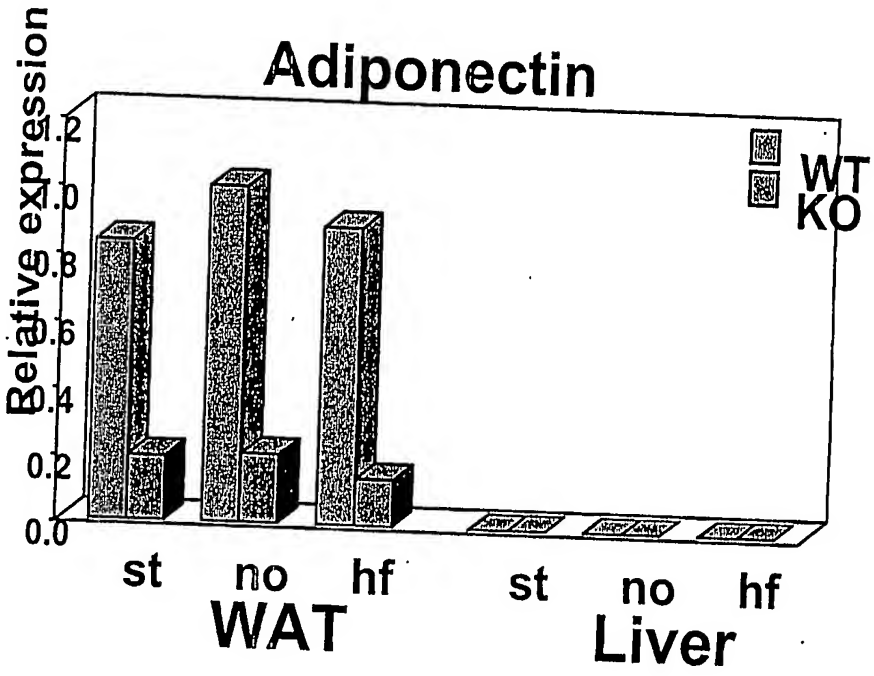

C/EBPb expression
Relative expression
WT
KO
0.0
0.5
st
no
hf
st
no
hf
WAT
Liver

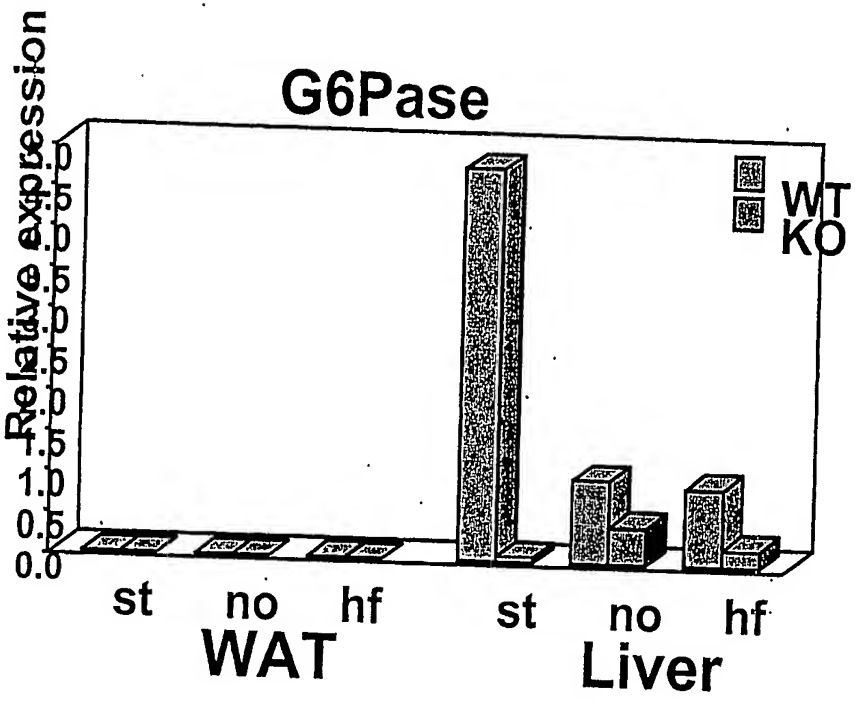

SREBP expression
Relative expression
WT
KO
0.0
0.2
0.4
0.6
st
no
hf
st
no
hf
WAT
Liver

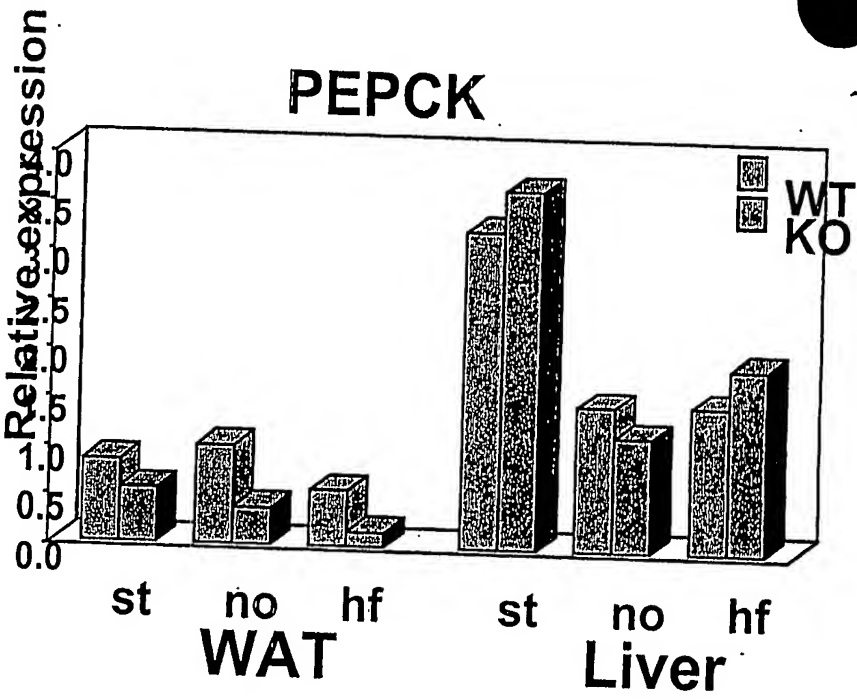

Glut4
Relative expression
0.0
0.2
WT
KO
st
no
hf
st
no
hf
WAT
Liver


aP2
Relative expression
0.0
0.2
WT
KO
st
no
hf
st
no
hf
WAT
Liver


Leptin
Relative levels
0.0
0.2
0.4
WT
KO
st
no
hf
st
no
hf
WAT
Liver


Adiponectin
Relative expression
0.0
0.2
WT
KO
st
no
hf
st
no
hf
WAT
Liver


G6Pase
Relative expression
WT
KO
st
no
hf
st
no
hf
WAT
Liver


PEPCK
Relative expression
WT
KO
st
no
hf
st
no
hf
WAT
Liver

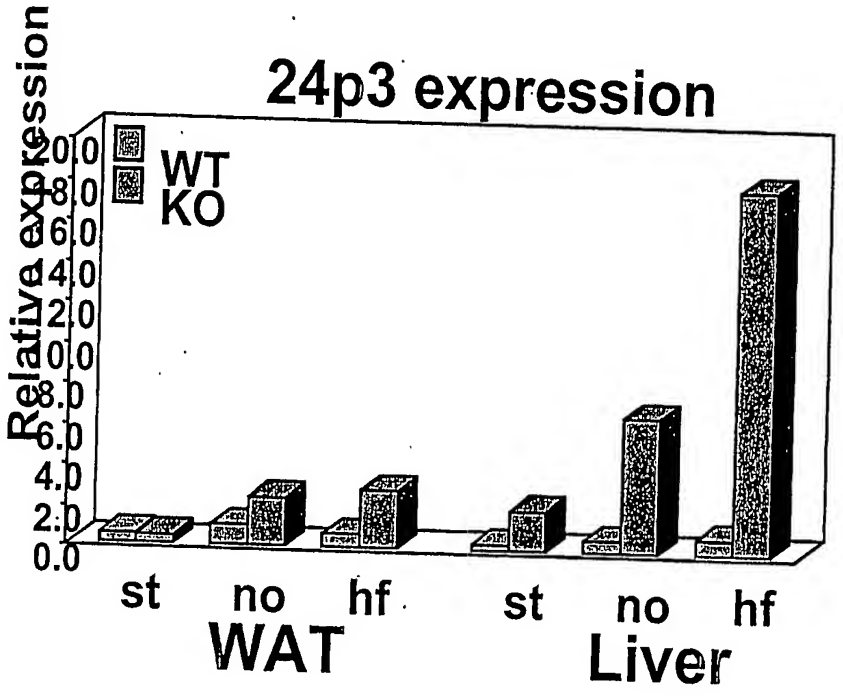
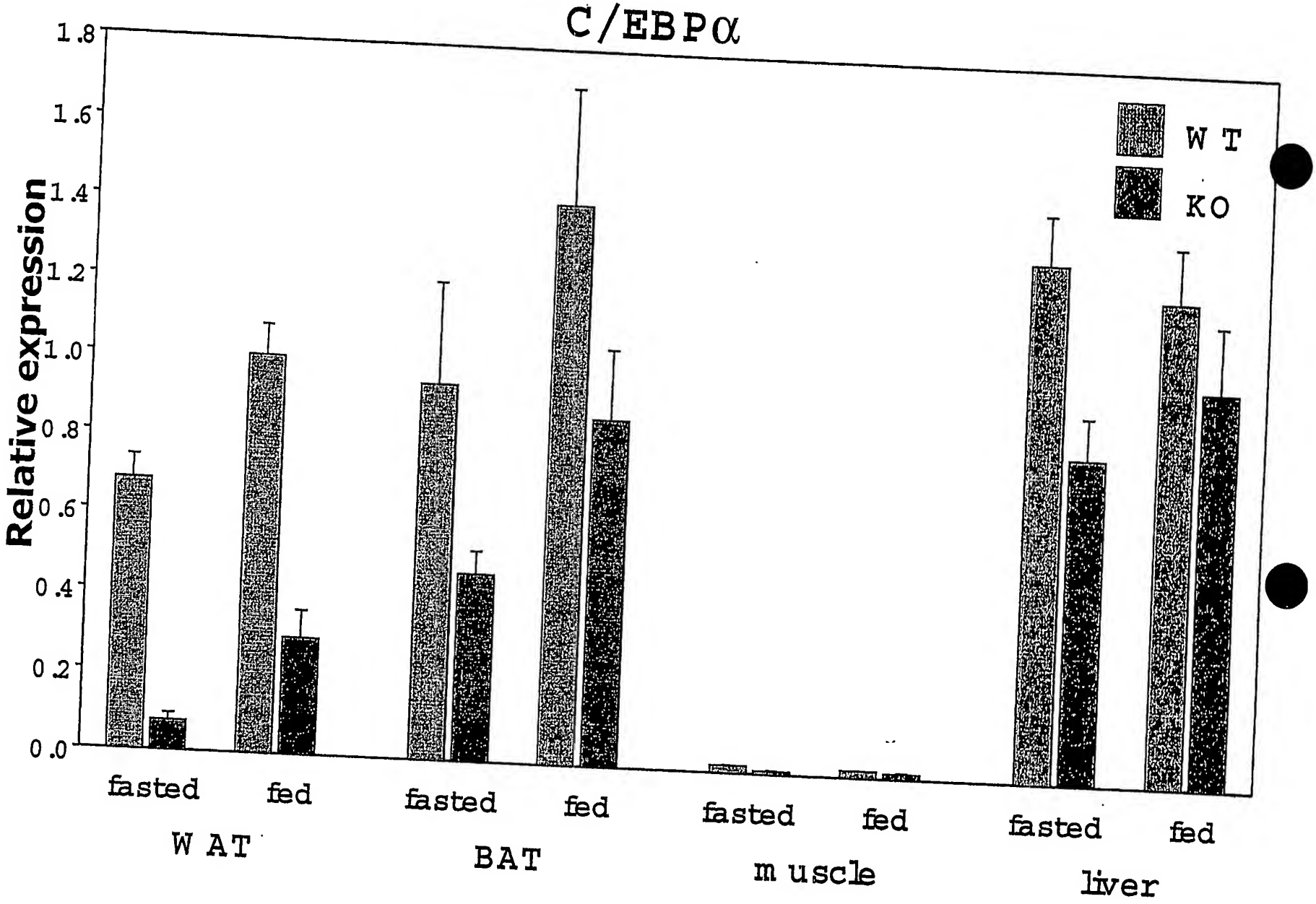

24p3 expression
Relative expression
WT
KO
st
no
hf
st
no
hf
WAT
Liver

FIG 1C


C/EBPα
Relative expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
KO
fasted
fed
WAT
BAT
muscle
liver

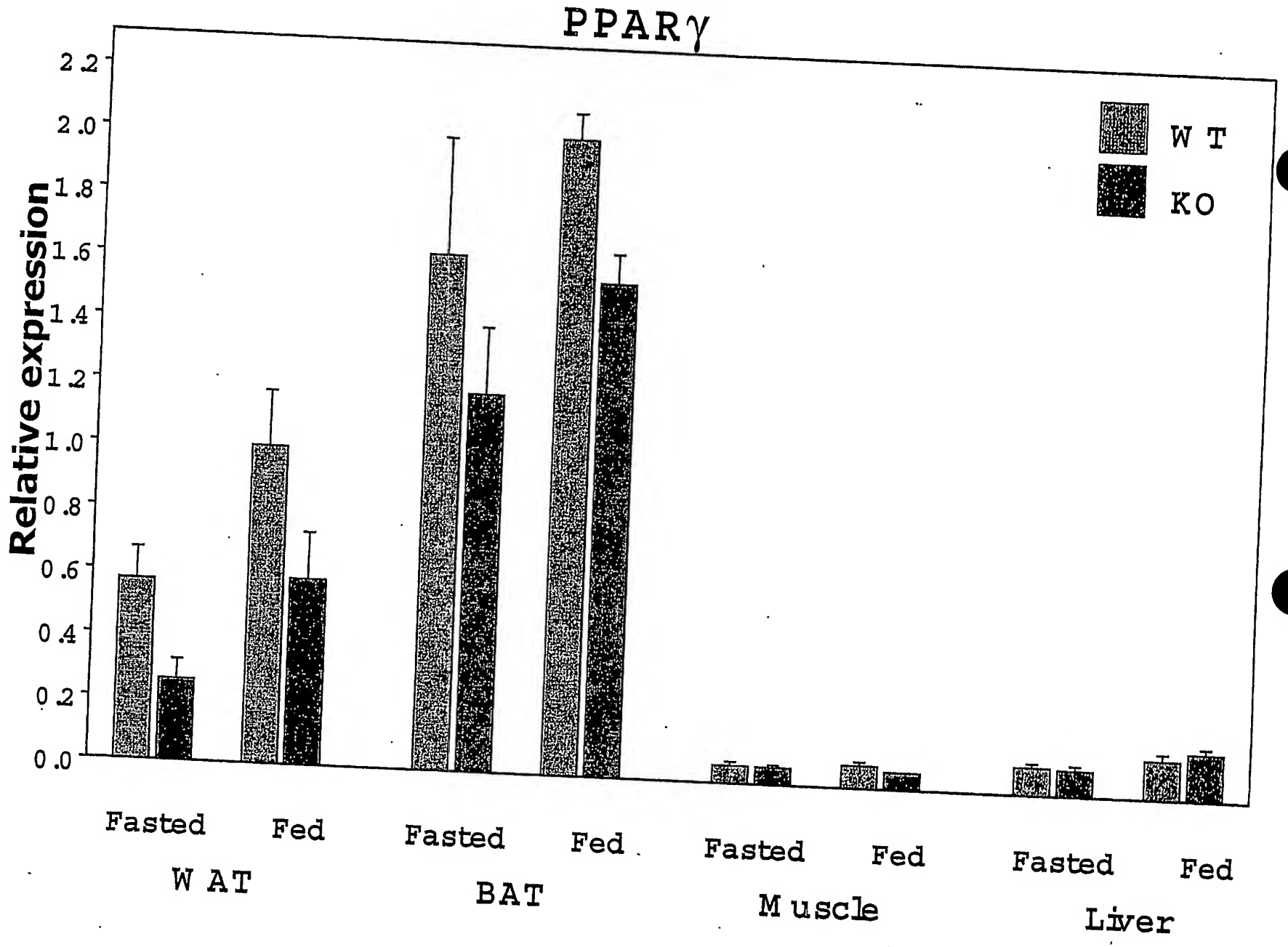

PPARγ
Relative expression
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
KO
Fasted
Fed
WAT
Fasted
Fed
BAT
Fasted
Fed
Muscle
Fasted
Fed
Liver

Fig 2

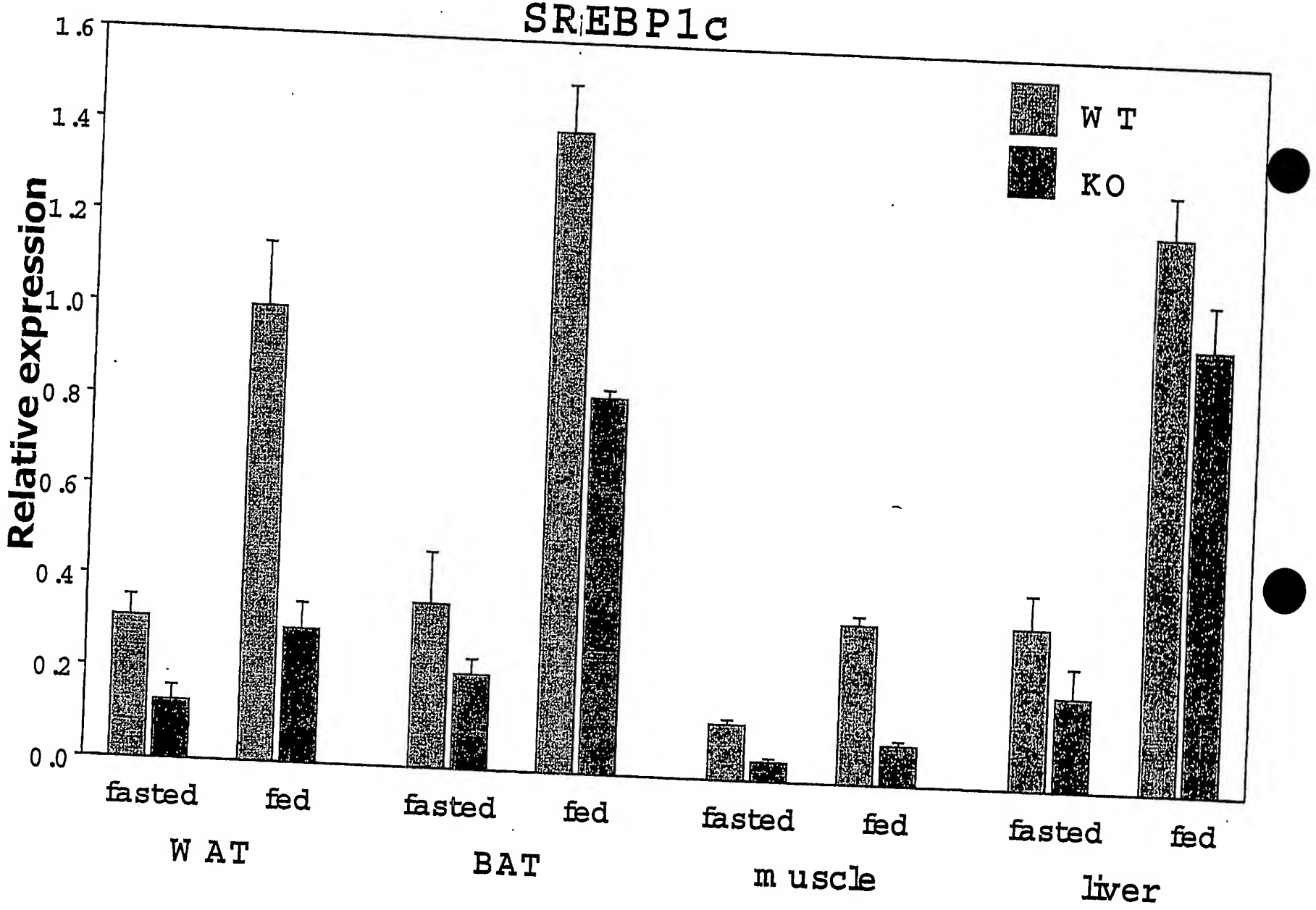

SREBP1c
Relative expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
KO
fasted
fed
WAT
BAT
muscle
liver

Fig 1

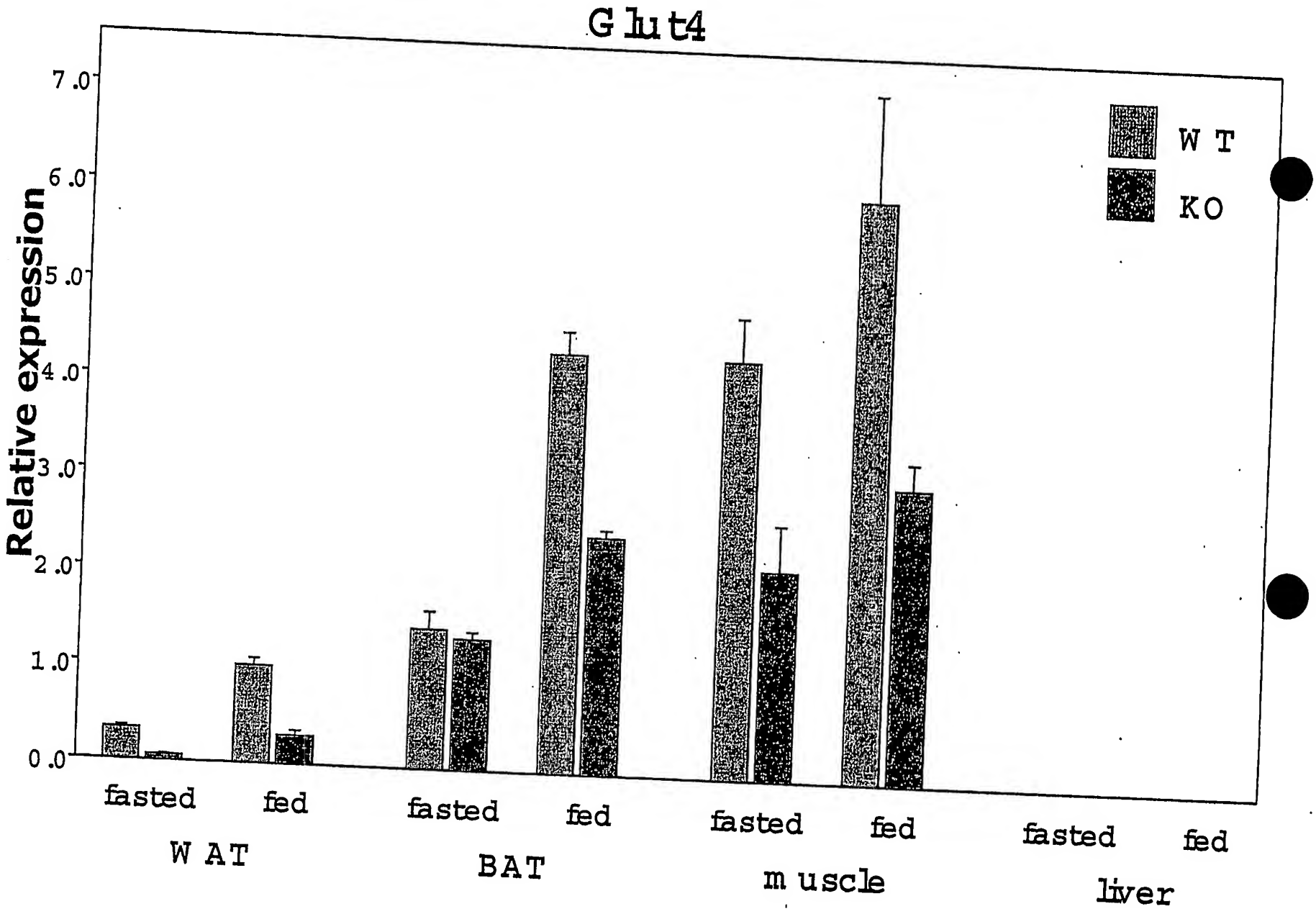
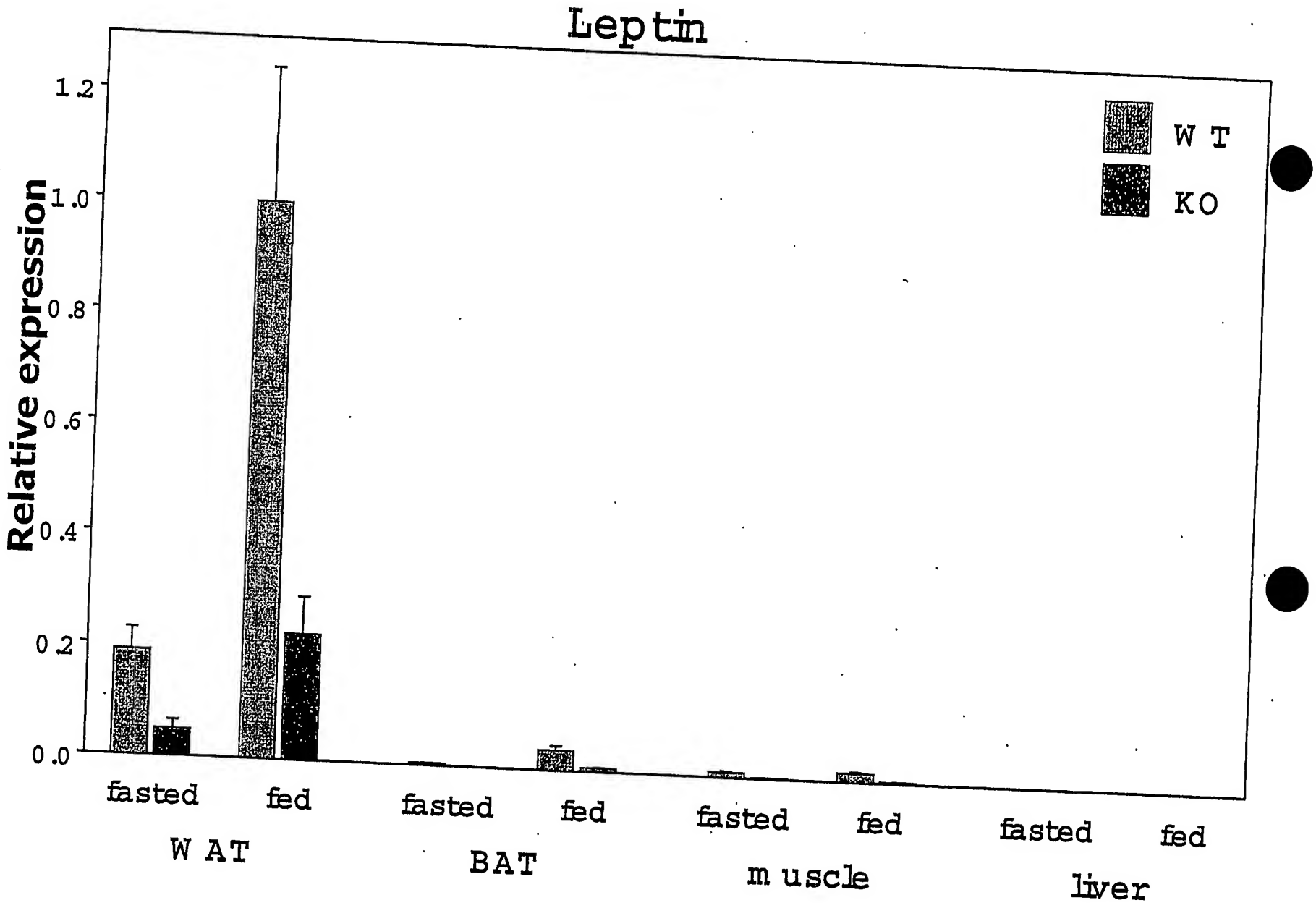

Glut4
Relative expression
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
WT
KO
fasted
fed
fasted
fed
fasted
fed
fasted
fed
WAT
BAT
muscle
liver

Fig 14


Leptin
Relative expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
KO
fasted
fed
fasted
fed
fasted
fed
fasted
fed
WAT
BAT
muscle
liver

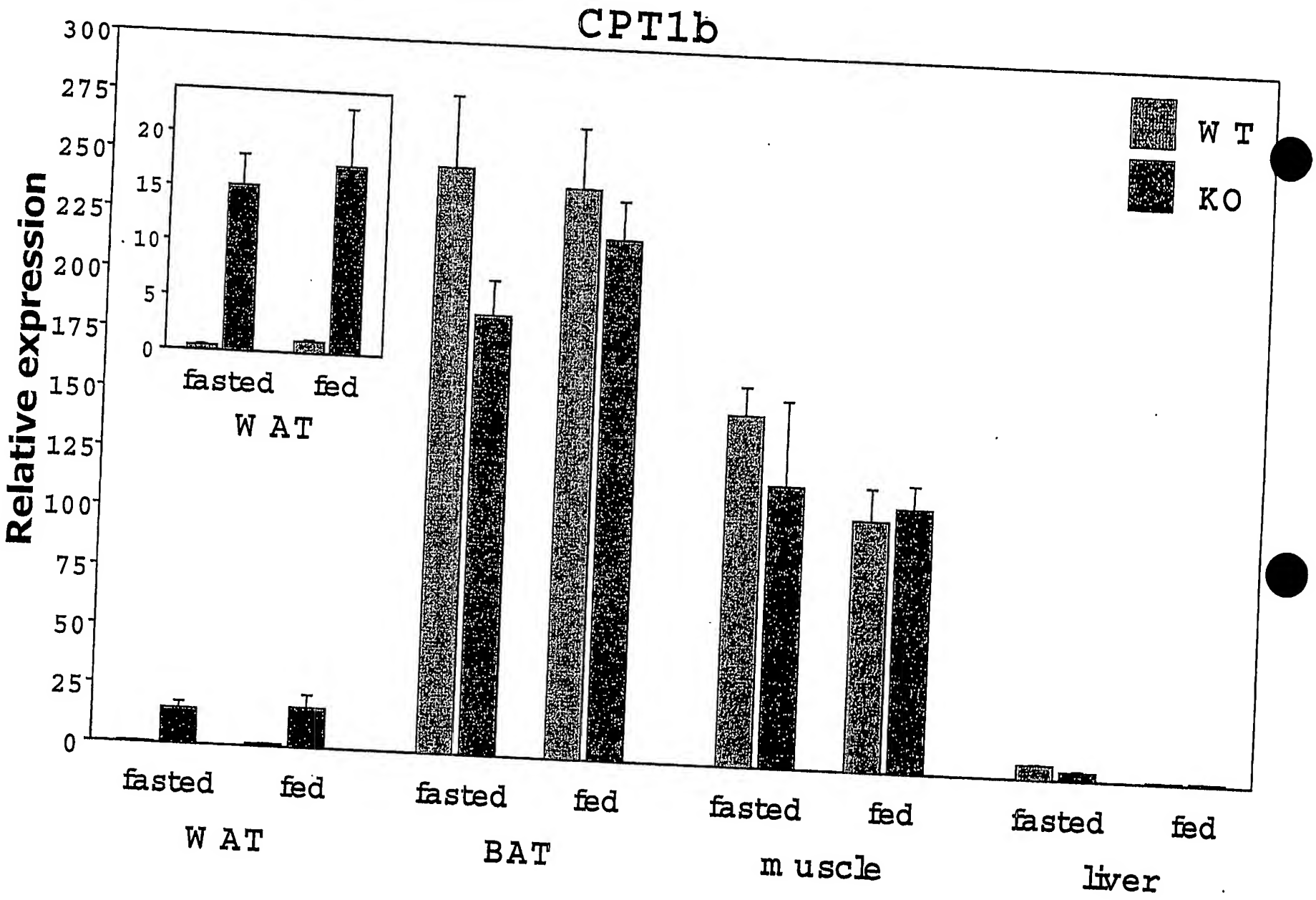

CPT1b
Relative expression
300
275
250
225
200
175
150
125
100
75
50
25
0
20
15
10
5
0
fasted
fed
W AT
WT
KO
fasted
fed
W AT
fasted
fed
BAT
fasted
fed
muscle
fasted
fed
liver

Fig 5

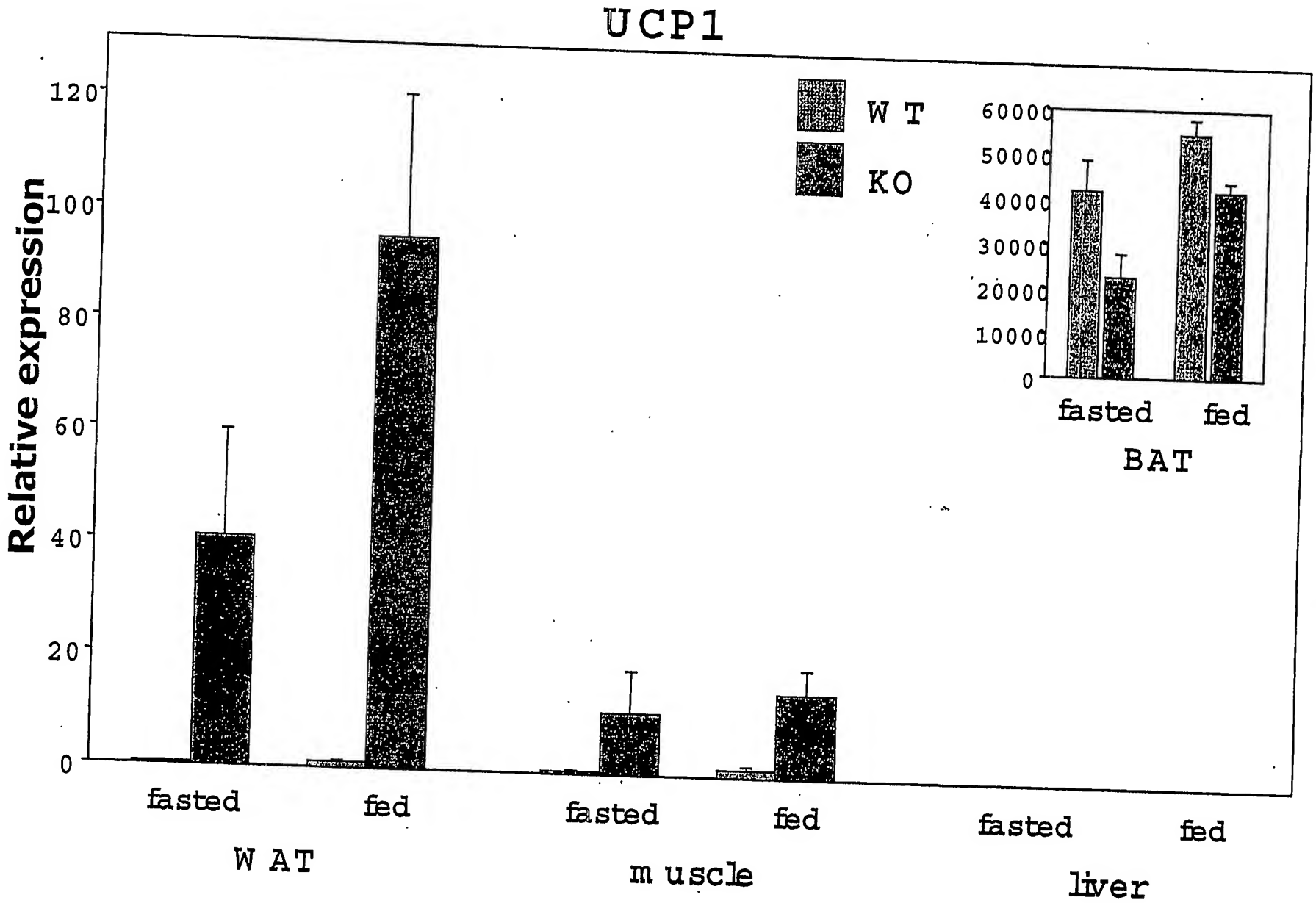
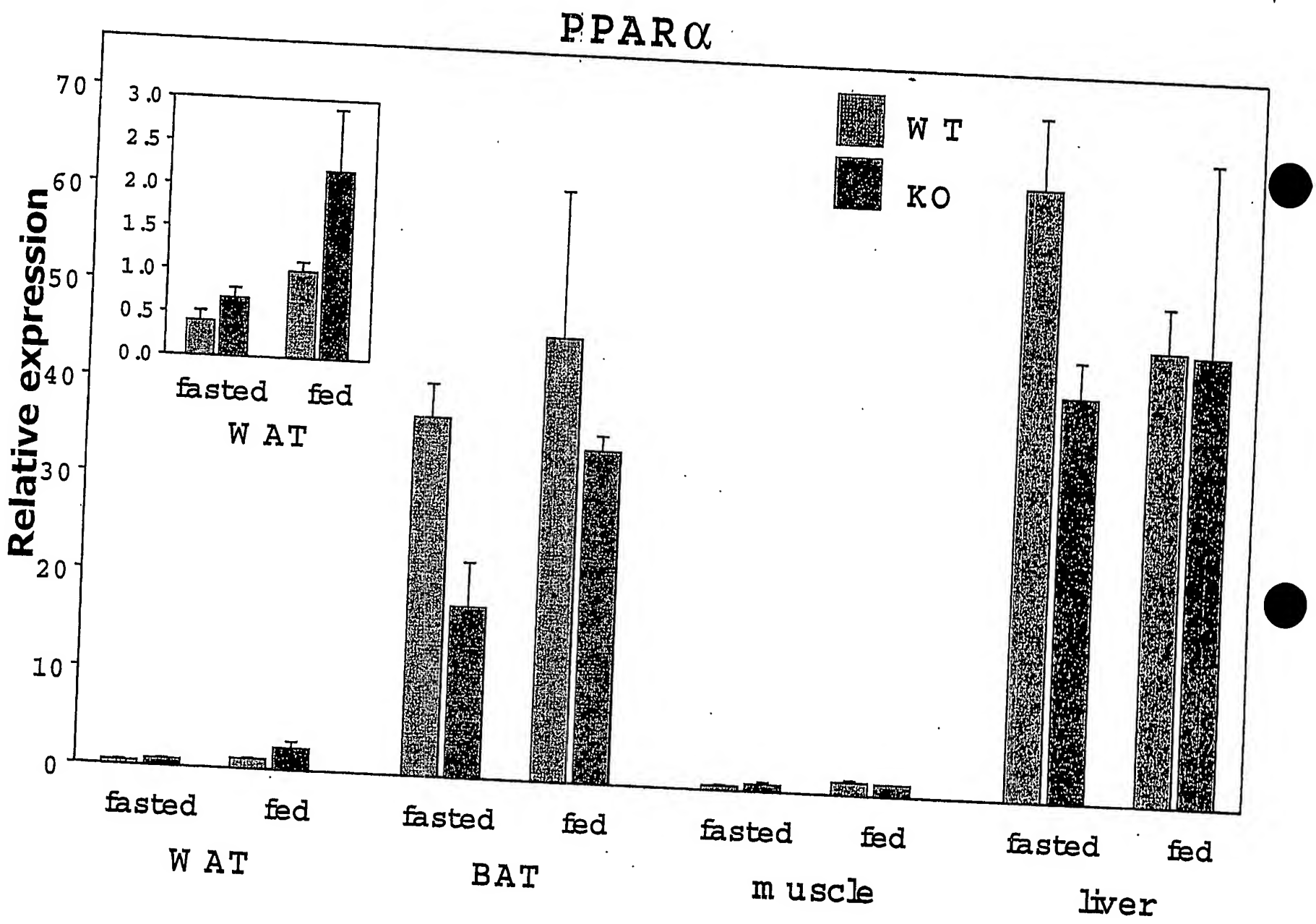

UCP1
Relative expression
120
100
80
60
40
20
0
WT
KO
60000
50000
40000
30000
20000
10000
0
fasted
fed
BAT
fasted
fed
WAT
fasted
fed
muscle
fasted
fed
liver

Fig 7


PPARα
Relative expression
70
60
50
40
30
20
10
0
WT
KO
3.0
2.5
2.0
1.5
1.0
0.5
0.0
fasted
fed
WAT
fasted
fed
WAT
fasted
fed
BAT
fasted
fed
muscle
fasted
fed
liver

Reduced triglyceride accumulation in liver tissue of Nrip1 null mice compared to wild type when maintained on normal [a] or high fat (35%) diet [b] for 10 days